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Factors Affecting Heme Transfer from "GAPDH": a Possible Heme Carrier Protein

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FACTORS AFFECTING HEME TRANSFER FROM “GAPDH”, A
POSSIBLE HEME CARRIER PROTEIN

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MASTER OF SCIENCE IN CHEMISTRY

at the

CLEVELAND STATE UNIVERSITY

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FACTORS AFFECTING HEME TRANSFER FROM “GAPDH”, A POSSIBLE HEME CARRIER PROTEIN

RAJESH VEMPATI

ABSTRACT

The aim of my research is to understand the mechanism of heme transfer by carrier proteins into heme proteins. The mechanism of heme insertion into proteins is poorly understood. Previous experiments reported from our lab identified GAPDH as a possible heme carrier protein involved in heme transfer (Ritu Chakravarti et al PNAS). This work focuses on 2 aspects of heme transfer. a.) Rate of transfer of heme from GAPDH to apomyoglobin. We examined the rate of transfer of native proto-heme and the analogue meso-heme. The results showed that the rate of heme transfer was twice as fast for meso-heme compared to the native proto-heme, for both human and rabbit GAPDH's. This suggests that structural factors may be important for heme transfer from GAPDH to apomyoglobin.

b.) Nitrosylation of GAPDH and its effect on rate of heme transfer. We went on to examine different factors that could affect the extent of nitrosylation. The factors which we examined were dose of nitrosylation donor, pH, and stability of nitrosylated protein with temperature and presence of oxygen. 1.) Increasing the dose of nitrosylation donor increased the extent of nitrosylation in the case of

human GAPDH, in contrary with rabbit GAPDH nitrosylation which is not dependent on dose 2.) Nitrosylated protein is more stable at 4oC than at room temperature. 3.) The extent of nitrosylation increased as the pH increases in case of rabbit GAPDH, however human GAPDH nitrosylation was favored at slightly acidic and alkaline compared to neutral pH. Reducing agents like DTT, tertiary amines like HEPES buffer and oxygen were found to have no effect on extent of nitrosylation. Previous cell biology work from our lab suggests that nitrosylation of Cys152 *in vivo* diminishes heme binding property of GAPDH. Cysteine residues that underwent nitrosylation were identified by mass spectrometry. The results from mass spectrometry suggest that the levels of nitrosylation on each individual cysteine are species-specific. In fact, rabbit and human GAPDH showed different levels of nitrosylation. In parallel site-directed mutagenesis was used to determine which one of the nitrosylated cysteine mutants had an effect on the rate of heme transfer. The results suggest that nitrosylation of wild type and different point mutants of GAPDH doesn't affect the rate of heme transfer, indicating that there is no substantial effect of S-nitrosylation on rate of heme transfer (Mild increase in rate of heme delivery for 2 cysteine mutants, C152/156S and C156S after nitrosylation).

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ABBREVIATIONS

GAPDH.....	Glyceraldehyde 3-phosphate dehydrogenase.
NOS.....	Nitric oxide synthase.
iNOS.....	Inducible NOS.
Hsp90.....	Heat shock protein 90.
ATP.....	Adenosine triphosphate.
NEM.....	N-Ethylmaleimide.
MMTS.....	methyl methanethiolsulfonate.
DTPA.....	Diethylene triamine pentaacetic acid.
EDTA.....	Ethylenediaminetetraacetic acid.
DMSO.....	Dimethyl sulfoxide.
HEPES.....	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

CHAPTER I

INTRODUCTION

1.1 Heme proteins:

Heme proteins are important proteins which play vital roles in physiology such as free radical elimination, signal transduction, oxygen transport, and electron transfer. Heme exists as a prosthetic group in several heme proteins, heme is an essential regulator of gene expression (1). The most common heme type is Fe-protoporphyrin which is heme B and others include A, C and O which are less common (2, 3). The last step in heme biosynthesis is the insertion of an iron atom into protoporphyrin which is catalyzed by ferrochelatase in the mitochondria (4, 5). Free heme is toxic to cells since it can cause damage to DNA, proteins and lipids by generating reactive oxygen species (ROS) therefore, cells keep tight control on free heme (6). Until now there is no confirmation how heme travels from mitochondria to heme proteins like myoglobin, hemoglobin, NOS and guanylate cyclase which are present in cytosol. A protein carrier involved in transporting heme from mitochondria to heme proteins has been proposed but yet

to be confirmed, until now there is no established pathway how heme gets incorporated into heme proteins.

1.2 Mechanism of heme delivery:

Previous studies in our lab about heme insertion into heme proteins such as myoglobin, hemoglobin and NOS suggest the role of carrier proteins in heme insertion (7). Previous research in our lab suggested that two proteins are important for heme insertion into apo-protein targets: Hsp90 and GAPDH. Heat shock protein (hsp90) plays a role in enabling heme insertion into cytokine inducible mouse NOS. Studies showed hsp90 was associated more with heme free iNOS, as compared to heme containing iNOS. Also, kinetic data suggested that cellular heme insertion coincided with hsp90 dissociation. The data also suggests that ATP deficient hsp90 blocked cellular heme insertion into heme free iNOS, Together, these observations suggest a potential role of hsp90 in cellular heme insertion into iNOS (8).

There are notable interactions between iNOS and GAPDH as determined by surface plasmon resonance, which suggests that GAPDH is a possible heme carrier protein to iNOS. It was also proposed that nitrosylation at Cys152 on GAPDH diminished the heme binding property of GAPDH (9).

GAPDH is one of the key enzymes in glycolysis. GAPDH catalyzes the conversion of glyceraldehyde-3-phosphate to high energy phosphate 1, 3-bisphosphoglycerate which is reversible and also first step in glycolysis (10).

GAPDH is involved in apoptosis, transcription activation, and may be heme carrier protein (11-14). GAPDH exists as a tetramer of 4 identical polypeptides each of 37kDa approximately. The human protein has 335-aminoacid, whereas in rabbit it is 333-aminoacid per monomer. Four subunits, which are designated as O, P, Q and R form the homotetrameric human liver GAPDH, which has 3 non-equivalent interfaces P, Q, and R. F_o-F_c difference map shows positive density around the active-site cysteine 152 of all 4 subunits. Rabbit skeletal muscle GAPDH is also very similar to human GAPDH as their sequence similarity was very high. The alignment shows 99% similarity between human and rabbit GAPDH which is represented in Fig.1.1

Accession numbers for the protein sequences:

Human GAPDH: P04406
Rabbit GAPDH: P46406

```

Query  2      VKVGVNGFGRIGRLVTRAAFN SGKVDVVAINDPFDLHYMVYMFQYDSTHGKFHGTVKAE  61
          VKVGVNGFGRIGRLVTRAAFN SGKVD+VAINDPFDL+YMVYMFQYDSTHGKFHGTVKAE
Sbjct  4      VKVGVNGFGRIGRLVTRAAFN SGKVDIVAINDPFDLNYMVYMFQYDSTHGKFHGTVKAE  63

Query  62     NGKLVINGKAITIFQERDPANIKWGDAGAEYVVESTGVFTTMEKAGAHKGGAKRVIISA  121
          NGKLVING ITIFQERDP+ IKWGDAGAEYVVESTGVFTTMEKAGAH+GGAKRVIISA
Sbjct  64     NGKLVINGNPITIFQERDPSKIKWGDAGAEYVVESTGVFTTMEKAGAHLQGGAKRVIISA  123

Query  122    PSADAPMFVMGVNHEKYDNSLKI VSNASCTTNCLAPLAKVIHDHFGIVEGLMTTVHAITA  181
          PSADAPMFVMGVNHEKYDNSLKI+SNASCTTNCLAPLAKVIHD+FGIVEGLMTTVHAITA
Sbjct  124    PSADAPMFVMGVNHEKYDNSLKIISNASCTTNCLAPLAKVIHDNFGIVEGLMTTVHAITA  183

Query  182    TQKTVDGPGSKLWRDGRGA AQNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPTPNVSVV  241
          TQKTVDGPGSKLWRDGRGA QNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPT NVSVV
Sbjct  184    TQKTVDGPGSKLWRDGRGALQNIIPASTGAAKAVGKVIPELNGKLTGMAFRVPTANVSVV  243

Query  242    DLTCRLEKAAKYDDIKKVVKQASEGPLKGILGYTEDQVVSCDFNSSATHSSTFDAGAGIAL  301
          DLTCRLEK AKYDDIKKVVKQASEGPLKGILGYTE QVVS DFNS THSSTFDAGAGIAL
Sbjct  244    DLTCRLEKPAKYDDIKKVVKQASEGPLKGILGYTEHQVVSSDFNSDTHSSTFDAGAGIAL  303

Query  302    NDHFVKLISWYDNEFGYSNRVVDLMVHMASKE  333
          NDHFVKLISWYDNEFGYSNRVVDLM HMASKE
Sbjct  304    NDHFVKLISWYDNEFGYSNRVVDLMAHMASKE  335

```

Query= rabbit GAPDH
Sbjct= human GAPDH

Fig.1.1. The above figure represents the alignment of human and rabbit GAPDH and the highlighted residues represent cysteines. The alignment was carried out using the blast tool at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>.

However there are certain differences as in case of human GAPDH each subunit is bound to NAD^+ and in the case of rabbit skeletal GAPDH only 2 subunits P and R binds to NAD^+ . The other difference is in their side-chain conformation of Arg234. Two residues, cysteine152 and histidine179 play major roles in catalysis. Cysteine152 provides a sulfhydryl group for nucleophilic effect on G3P and histidine179 functions as a base catalyst to facilitate hydride transfer (15, 16). Our lab has found that the catalytic properties of GAPDH are not affected by heme binding (Unpublished data).

Human GAPDH crystal structure

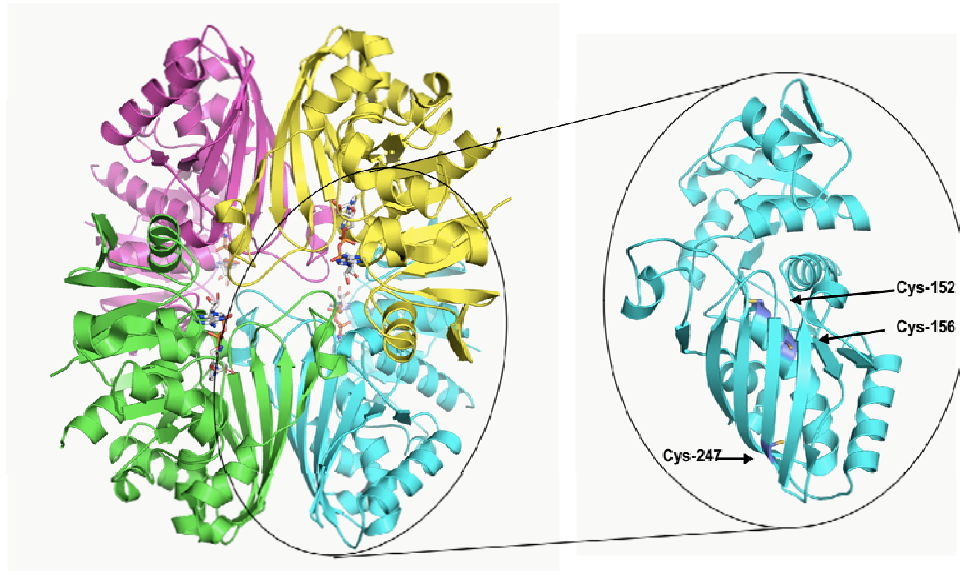


Fig.1.2. Crystal structure of human GAPDH, tetramer protein having 4 identical monomers. Each monomer has 3 cysteines which are shown above at 152, 156 and 247. Cysteine 152 is responsible for GAPDH activity.

Rabbit muscle GAPDH crystal structure

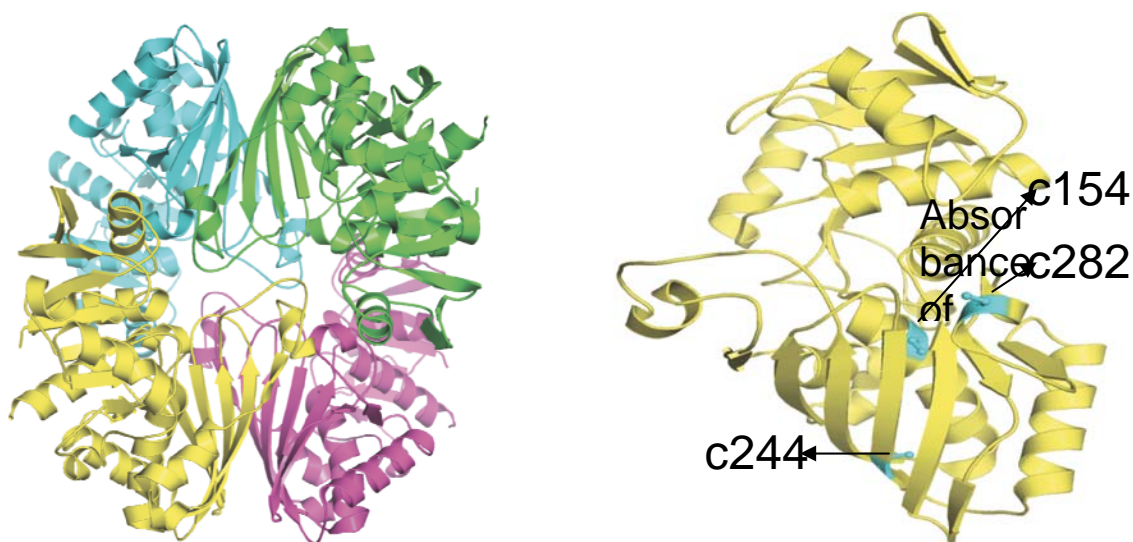


Fig.1.3. Crystal structure of rabbit muscle GAPDH, Tetramer having four identical monomers and having close similarity to human GAPDH. Four cysteines are present at 150, 154, 244 and 282. Here cysteine 150 is not shown.

This project deals with rate of heme transfer from GAPDH to apomyoglobin. We set up an assay to determine the rate of heme transfer from GAPDH heme complex to Apo myoglobin. We considered different factors, which may have an effect on the rate of heme transfer from GAPDH-heme complex. The mutant Apo myoglobin glycerol stock was obtained from Dr. John S. Olson (Rice University, Texas, USA).

Myoglobin is a monomeric heme protein which is present mainly in muscle tissues because of its ability to bind oxygen and store it. It is a single chain globular protein with 154 aminoacids containing heme prosthetic group in its center. The molecular weight of our mutant is 17.4kDa. It is made of 8 alpha helices joined by

short loops, and is water soluble. Each myoglobin has 1 heme prosthetic group inserted into a hydrophobic cleft in protein. The hydrophobic interactions between hydrophobic aminoacid group present in the interior side of the cleft and tetrapyrrole ring stabilize the heme binding to protein (17, 18). Heme binding to the protein is also stabilized by an axial nitrogen atom provided by histidine 93 at 2.09 angstroms from iron-heme; the iron is also chelated by histidine 64. Our lab use a mutant form of apo-myoglobin that has an increased affinity for heme (molecular weight =17.4 K Da). In the mutant apomyoglobin, His 64 is replaced with Tyr to increase its affinity for heme. Val68 is replaced with phenylalanine which makes it stable (19, 20).

Crystal Structure of Sperm whale myoglobin

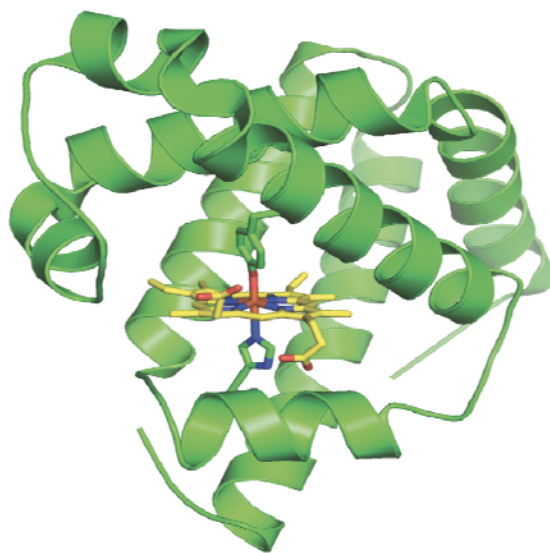


Fig.1.4. Crystal structure of sperm whale myoglobin, Histidine 64 has been replaced by tyrosine to increase the affinity towards the heme. The heme is represented in yellow. All crystallographic images were generated with program pymol education edition.

The two types of heme for which we studied the rate of heme transfer are proto-heme which is native and the meso-heme, which differs on the side chains (R^1) as shown below (21) in Fig.1.5.

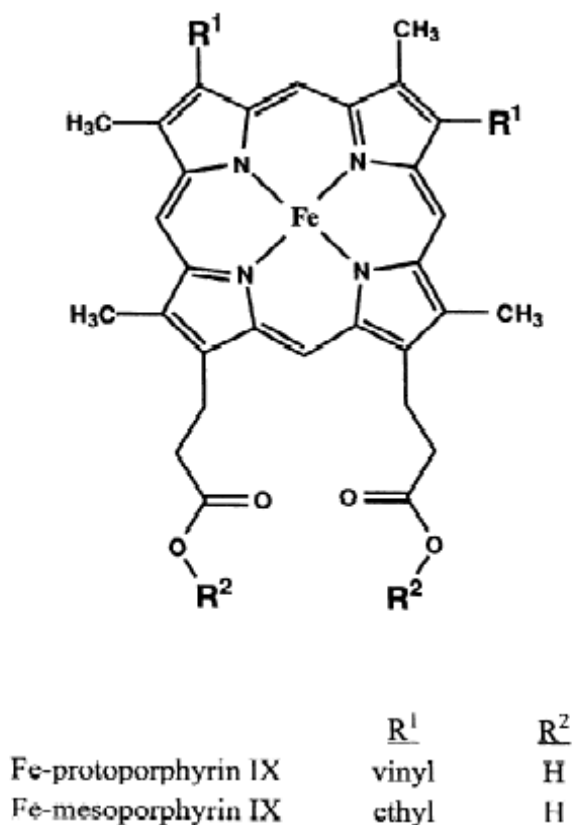


Fig.1.5. Structure of Fe-proto and mesoporphyrin IX .

Human GAPDH has a total of 3 cysteines at positions 152, 156 and 247, rabbit GAPDH has total of 4 cysteines at 150, 154, 245 and 282 respectively. Previous work from our lab suggests that heme insertion is regulated by NO via specific nitrosylation of GAPDH in vivo. This project explores an in vitro model to identify which cysteine is involved in nitrosylation. In addition, we investigate

whether a correlation exists between the nitrosylation of specific cysteine and the rate of heme transfer to apomyoglobin. We determined the extent of nitrosylation of each specific cysteine on GAPDH by mass spectrometry.

1.3 Nitrosylation:

Nitrosylation of proteins is becoming an important physiological process involved in major role in host defense, neurotransmission and DNA repair. The transfer of NO group (nitric oxide) from NO donor to free SH group which are present on proteins leading in formation of nitrosothiols (RSNO) is known as nitrosylation of proteins (22). Thiol groups play an important role in quaternary structure and co-factor binding. Free SH group is a good biological nucleophile and is good targets for electrophiles and also for redox agents which includes nitrosylating and oxidizing species generated from NO. S-nitrosylation influences protein function such as enzyme inhibition by blocking active site which is Cys 152. The cysteines are the main targets for nitrosylation. In general RSNO bond is unstable and hence it is difficult to quantify (23).

The physiological RSNO's includes S-nitrosoglutathione (GSNO) and S-nitroso-L-cysteine which have been detected and quantified in vivo (24). Protein S-nitrosylation has inhibitory effects on blood clot formation and it is demonstrated that factor XIII is target for S-nitrosylation on a highly reactive cysteine residue which has inhibitory effect on blood coagulation and so RSNO's plays a major role in hematology (25). S-nitrosylation of GAPDH prevents cytotoxicity by

binding with the novel protein GOSPEL (26). Nitrosylation is specific which means that every free SH group is not nitrosylated and if nitrosylated, nitrosylation may not be to same extent (27). In our target protein human GAPDH we have 3 cysteines in total at positions 152, 156, and 247 in the primary structure. As per the results of mass spectrometry we observed that extent of S-nitrosylation varies on each cysteine. S-nitrosylation inactivates GAPDH, and is reversible as S-nitrosylated GAPDH is unstable as SNO bond is liable (28).

Biotin switch technique:

S-nitrosylation of proteins is continuously emerging as physiologically important modification by which many cell functions are monitored. It is important to determine which free SH group undergoes nitrosylation and also to determine the extent of nitrosylation on each individual free SH group. The principle in biotin switch includes blocking of free SH groups by using thiol blocking agents like MMTS (methyl methane thiosulfonate) or NEM (N-ethylmaleimide) after nitrosylation so that only Nitrosylated cysteines are available for biotin in next step. The next step is reducing the S-Nitrosylated thiol with ascorbate and finally the reduced form is labeled with Biotin-HPDP. The amount of nitrosylation which is in terms of biotinylation can be determined by either immunoblotting or by using mass spectrometry (29, 30).

Modified biotin switch method: This is a slight modification of biotin switch technique. This has high sensitivity in quantitative estimation of nitrosylation on

each individual free thiol group in a protein. Here we replace normal thiol blocker MMTS with NEM and iodoacetamide for alkylating nitrosylated cysteines instead of biotin-HPDP.

CHAPTER II

EXPERIMENTAL METHODS

2.1 Preparation of heme solution:

The heme solution was prepared by dissolving 10 mg hemin (MP biomedical pdt # 02198820) in 4.5 ml water, add little 1 M NaOH (Fisher Scientific# s318-1 CAS 1310-73-2), until visually dissolved. Spectra were collected from 700-300nm using a Shimadzu UV-VIS spectrophotometer, NaOH was added until consecutive peaks at 385 nm remain unchanged. Then filter the heme solution through 0.22 micron filter and collect the filtrate. The concentration of heme was measured by the heme-chromogen assay.

The heme-chromogen assay was performed using Shimadzu UV-VIS spectrophotometer. The heme-chromogen reagent was prepared by mixing 400 μ l pyridine, 400 μ l water and, 1M 200 μ l NaOH. 5 μ l heme solution (dilution depends on concentration of heme solution) was added to 495 μ l heme chromagen reagent, mix with dithionite and incubate for 2-5 minutes. The baseline was taken with heme chromagen reagent in the cuvette. Spectra were collected from 700-400

nm. The peak height at 556 nm was measured, using extinction coefficient of 34600/ M CM (31).

2.2 Preparation of GAPDH-heme complex:

2mg GAPDH from rabbit muscle (sigma PDT# G2267) was dissolved in Hepes (Fisher Scientific# BP 310-500 CAS 7365-45-9) 50mm/100mm NaCl (NPI CAS# 7647-14-5 >99% pure)/pH 7.4, to achieve a final concentration of 200 μ M. Then the protein solution was incubated with 300 μ m heme for 30-45 min at room temperature. Disposable PD-10 desalting columns (GE health care PDT Code# 17-085-01), which were pre packed with sephadex G-25 medium. These were used to remove unbound small molecules from large molecules like proteins. These PD-10 columns were washed with 5-7 column washes water and equilibrated with 50mm HEPES/ 100mm NaCl/ pH 7.4. The GAPDH-Heme complex was then passed through these PD-10 columns. This Pd-10 gel filtration step removes free heme from protein-heme complex. The fractions each of 500 μ l were collected in 1.5 ml centrifugal tubes and protein concentration of fractions was determined, fraction containing GAPDH-heme complex was used for further analysis.

2.3 Estimation of Protein concentration:

The concentration of protein was determined by taking spectral scan from 700-250 nm and by measuring peak height at 280 nm using UV-VIS spectrophotometer.

The extinction coefficient used for GAPDH was 30000/ M CM. The extinction coefficient used for apomyoglobin was 15200/M CM.

2.4 Koff or transfer of heme from GAPDH from GAPDH-heme complex to apomyoglobin:

We established an *in vitro* system to study the transfer of heme from GAPDH-heme complex to apomyoglobin. Multiskan spectrophotometer was used to measure transfer rates that can read 96wells at same time, this is used to conserve time and material. The principle involved here, is to monitor the spectral changes that occur upon heme transfer from GAPDH-heme complex to apomyoglobin. Spectral changes recorded for 90 minutes with 30 second interval, heme transfer from GAPDH-heme complex to apomyoglobin is irreversible. 20 μ M GAPDH of 200 μ L was taken into each well and surrounding wells were filled with water to prevent evaporation, 40 μ M apomyoglobin was added into 3 sample wells, no Apo myoglobin into control wells. Spectral changes at 398 nm and 700 nm was recorded for Fe-mesoporphyrin IX and 411 nm and 700 nm for Fe-protoporphyrin IX. Subtracting 398 nm from 700nm and sample from control wells for Fe-mesoporphyrin IX, and 411 nm from 700 nm and sample from control for Fe-protoporphyrin IX, rates of heme transfer were calculated by fitting to exponential. Hsp90 and cell lysate proteins effect on the rate of heme transfer to apomyoglobin. The compositions of different mixtures for each reaction well are listed below.

GAPDH human-proto heme Complex concentration into each well =10 μ M.

Apomyoglobin concentration into each well	=20 μ M.
Cell lysate mix into each well	= 4.95 mg/ml.
Cell lysate 50k and more into each well	= 4.45 mg/ml.
Cell lysate 50k and more into each well	= undetectable.
Hsp90 alpha into each well	= 2.52 μ M.
Hsp90 beta into each well	= 12.52 μ M.
ATP into each well	= 1.6 mM.

2.5 GAPDH activity:

GAPDH activity was determined by measuring the changes in absorbance at 340 nm which is the absorbance for NADH. Assay mixture for activity contains 82.3 mM triethanol amine (Sigma# T9534-250g 99.5 % pure) buffer pH 7.6, 1.1 mM ATP (Sigma# A2383-10 g), 6.2 mM glycerate 3-phosphate (Sigma# P8877-1g 99 % pure), 0.2 mM NADH (Sigma# N-8129 98% pure), 0.9 mM EDTA (Sigma# E5134-1 kg), 2 mM MgSO₄ (Sigma# M7774) and phosphoglycerate kinase (13 units/ml) (Sigma# P7634-2 ku) (14).

Kinetics of NADH disappearance was measured by using Shimadzu UV-VIS spectrophotometer at 340 nm. 0.1-0.5 μ M of GAPDH was taken into the cuvette containing 495 μ l of activity mixture and the activity was calculated by measuring the rate of change of absorbance per minute at 340 nm, where NADH is oxidized into NAD. 1 unit of GAPDH oxidizes 1 mole of NADH. Enzymatic activity was

calculated from the slope and by using extinction coefficient 6.22/ M CM (NADH at 340 nm).

2.6 Preparation of CYS-NO:

CYS-NO was prepared by taking 200 mM L-cysteine (Aldrich# 168149-25 g 97 % pure) in 200 μ M DTPA (Fluka# 32319-100 g-F.) and 200 mM HCl of 500 μ l mixed with 500 μ l sodium nitrite (Sigma#s-2252-500 g) solution of 210 mM. The solutions were mixed and incubated in the dark for 10 minutes. The pH was adjusted to 8 with NaOH. The concentration was measured at wavelength 336 nm by Shimadzu UV-VIS spectrophotometer by using an extinction coefficient of 900 /M CM.

2.7 Preparation of SNO-GAPDH:

500 μ l of 100 μ M of recombinant human GAPDH were made in 50mM HEPES, 100 mM NaCl, 75 μ M DTPA, pH 7.4. 50 fold CYS-NO was added and incubated for 30 minutes. Passed it through PD-10 columns made of G25 resin and fractions were collected and the protein concentration was determined by measuring the absorbance at 280 nm on a Shimadzu UV-VIS spectrophotometer by using extinction coefficient 30000 /M CM.

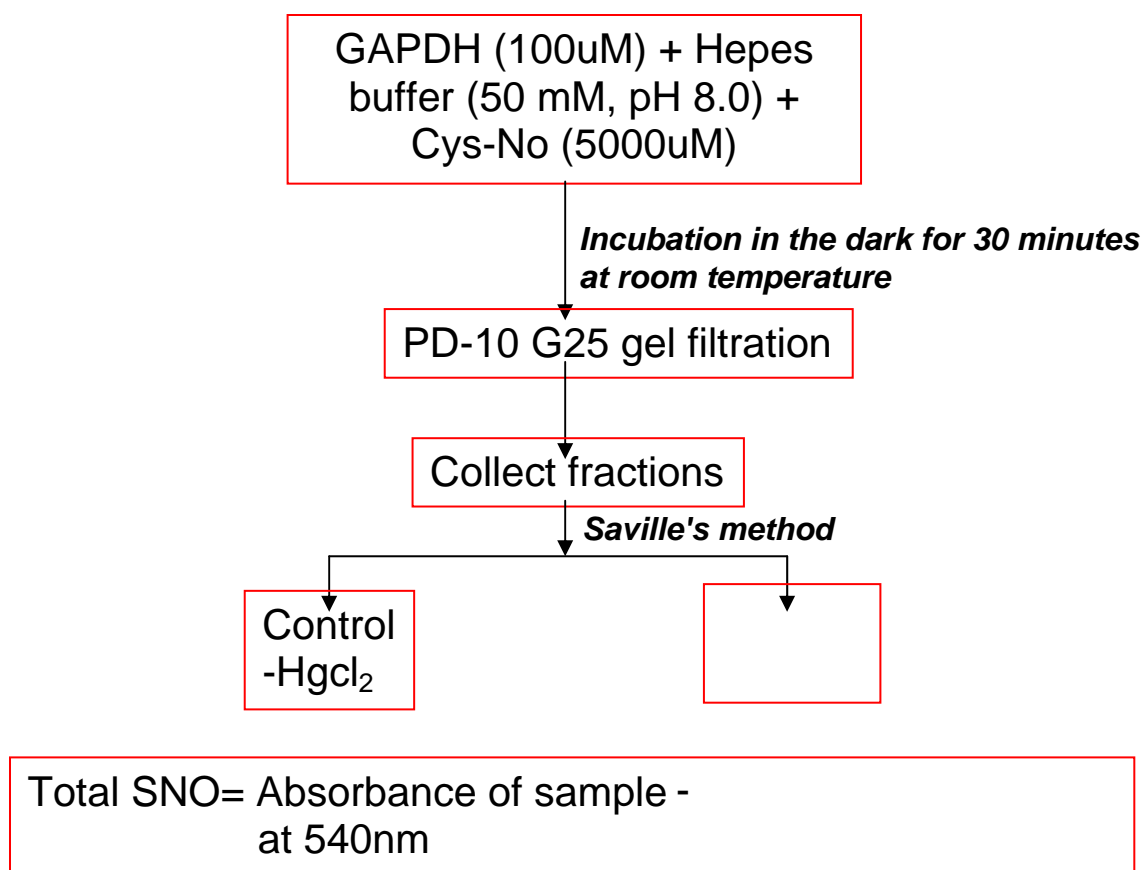


Fig.2.1. Schematic representation of the method to generate nitrosylated GAPDH and its quantification.

2.8 Saville's method:

A colorimetric assay was used for the quantification of SNO. HgCl₂ displaces NO and the NO was made to react with Griess reagent. Griess reagent includes 3.4% W/V sulfanilamide (Sigma# S-9251.) in 0.4 M HCl and 0.1% W/V of N-(1-naphthyl) ethylenediamine (NED) (Sigma# N-9125.). HgCl₂ (Sigma# M-6529) was mixed into sample and not into control. The difference between HgCl₂ absorbance reading and without HgCl₂ absorbance reading gives the amount of SNO. The absorbance was measured at 540 nm. The exact procedure includes 100

μ l of nitrosylated protein sample and 50 μ l 3.4% W/V sulfanilamide with HgCl_2 10 mM and 50 μ l 0.1% W/V of N-(1-naphthyl) ethylenediamine (NED) and control includes the same without 10 mM HgCl_2 . The detection limit was 500 nM. A summary of this method was provided in Fig.2.1.

The standard curve was made by using nitrite solution of different concentration 1 μ M, 2.5 μ M, 5 μ M, 7.5 μ M, 10 μ M, 12.5 μ M, 15 μ M, 20 μ M, 25 μ M, 50 μ M, 75 μ M, 100 μ M. The slope was calculated and by using slope, amount of SNO on protein was calculated from the absorbance at 540 nm.

2.9 Treatment of the protein with DTT:

To reduce disulphide bonds the protein was incubated with DTT (Dithiothreitol). In our studies for nitrosylation of proteins essentially there should not be any disulphide bonds. We treat proteins with 10 fold excess of DTT for 1.5-2 hours and the excess DTT is removed by passing it through PD-10 column which are previously washed and equilibrated with buffer (32).

2.10 Effect of pH and oxygen on nitrosylation:

Degassing of buffers and reagents:

All the buffers, NaOH, DTPA solutions were degassed by using nitrogen gas for 2 hours.

Placing the materials inside Anaerobic Chamber:

PD-10 columns, L-Cysteine, sodium nitrite, DTPA/HCL solution, Rabbit GAPDH lyophilized powder, Human C152S mutant GAPDH, 1M NaOH, HEPES 50mM/100 mM NaCl/75 μ M DTPA buffers with pH 6, 7, 8. Griess reagent, HgCl₂, 1.5 ml centrifugal tubes, cuvettes, 96 well plates, water were placed in vacuum port for degassing by using nitrogen gas and then in anaerobic chamber. The Cys-NO and SNO-GAPDH was made in anaerobic chamber.

Note: Everything has to be done in the dark as SNO bond is light sensitive and liable by light.

2.11 Preparation of GAPDH at different pH:

A solution of 100 μ M Human C152S GAPDH and Rabbit wild type GAPDH were made in 50 mM HEPES, 100 mM NaCl, 75 μ M DTPA in 3 different pH ranges 6, 7, and 8 by measuring concentration at wavelength 280 nm by Shimadzu UV-VIS spectrophotometer by extinction coefficient 30000/M cm.

2.12 Site directed mutagenesis:

Cysteines in GAPDH were replaced with serines to identify site of nitrosylation and its effect on heme binding and heme delivery. There are a total of 3 cysteines in human GAPDH at 152, 156 and 247 and total of 4 cysteines in rabbit GAPDH at 150, 154, 245 and 282 positions. The mutations which were considered were single mutations C152S, C247S and double mutations C152/156S, C152/247S, C156/247S and a triple mutant C152/156/247S in which all the cysteines were

replaced with serines. We consider serine as replacement because serine has near equal molecular size as cysteine. The required DNA sequencing primers were obtained from IDT.

2.13 Polymerase chain reaction:

This is a molecular biology technique which is used for the amplification of DNA to generate millions of copies of particular DNA sequence. It involves repeated cycles of heating and cooling for DNA melting and DNA replication. The 2 complementary oligonucleotides which have the desired sequence or mutation were obtained from IDT. The PCR mixture has the following 5 μ l of 10X reaction buffer, 10-20 ng ds DNA template, 125 ng of oligonucleotide primer 1, 125 ng of oligonucleotide primer 2, 1 μ l dNTP mix and make into 50 μ l with ddH₂O. 1 μ l of pfu turbo DNA polymerase (2.5 U/ μ l) was added. The dNTPs were the building blocks from which new DNA strand is synthesized by DNA polymerase. The quick change XL site directed mutagenesis kit was obtained from Stratagene (Stratagene# 200517-5).

The robocycler for PCR was set up by using following the program a.) Segment 1 has 1 cycle at 95⁰ C for 30 seconds. b.) Segment 2 has 18cycles each at 95⁰ C for 30 seconds, 55⁰ C for 1 minute and 68⁰ C for 10 minutes. Segment 3 has 0 cycles and every temperature for 0 seconds. Segment 4 has 6⁰ C, set up for overnight, and after reaction was done, it stays at 6⁰ C. DPN1 was added finally to PCR mixture

and incubated at 37⁰ C for 2 hours to degrade template DNA, the PCR mixture was transformed into XL 10 Gold ultra competent cells.

2.14 Transformation into XL10-Gold ultra competent cells:

The XL10 Gold ultra competent cells (Cat# 200315) were obtained from stratagene. Two 14 ml BD falcon tubes were chilled on ice and NZY broth was heated to 42⁰ C. The XL-10 cells were thawed on ice and 50 µl of cells placed in each of the tubes and add 4 µl 2ME, swirl every 2 minutes for 10 minutes by placing on ice. 2 µl of PCR mixture was added and incubated on ice for 30 minutes, Heated in water bath at 42⁰ C, incubated on ice for 2 minutes. 0.5 ml preheated NZY broth was added to the mixture and incubated at 37⁰ C for 1 hour shaking at 250 rpm. 250 µl of cells were plated on pre warmed LB-Amp agar plates and incubate overnight at 37⁰ C. The colonies were sequenced to confirm the presence of mutation.

2.15 DNA purification:

Up to 6 colonies were picked from each plate and grown in LB-Amp overnight. The cells were harvested in 6 different centrifugal tubes by spinning down at 13000 rpm for 2-3 minutes and the supernatant was discarded. Cells were suspended in 250 µl P1 buffer to which RNase was added previously. 250 µl P2 buffer was added and mixed, 350 µl N3 buffer which is neutralization buffer was added and mixed. Centrifuged for 10 minutes at 13000 rpm and supernatant was

applied to QIA prep spin column and flow through was discarded as our plasmid DNA was bound to column. Washed with 0.75 ml washing PE buffer and centrifuged for 60 seconds. Flow through was discarded and centrifuged for 1 minute to remove any remaining PE buffer. The last step was the elution of the DNA. Clean autoclaved 1.5 ml centrifugal tubes were taken and QIA prep column (Qiagen# 27106) was placed into them, 50 µl elution buffer was added to column and centrifuged for 1 minute. The plasmid DNA was eluted into centrifugal tube.

2.16 Transforming DNA into over expressing cells:

The plasmid DNA carrying the desired mutation was transformed into over expressing BL-21 cells. 1.5 ml C-medium was heated to 37⁰ C and 0.15 ml of overnight culture was added to 1.5 ml C-medium which was at 37⁰ C and left in shaker for 20 minutes at 37⁰ C. Transform aid T-solution was prepared by mixing 250 µl of T-solution (A) and 250 µl T-solution (B) (Fermentas# K2711). The culture (C-medium) was spun down at 13000 rpm for 2 minutes and the pellet was suspended in 300 µl transform aid T-solution, incubated on ice for 5 minutes. Centrifuged, supernatant was removed and 120 µl transform aid T-solution was added and incubated on ice for 5 minutes. 1 µl DNA was added into 50 µl cells in transform aid T-solution and incubated on ice for 5 minutes. Cells were plated on LB-Amp agar plates which were pre warmed at 37⁰ C and incubated overnight at 37⁰ C, control was performed by plating 50 µl cells from transform aid T-solution without adding plasmid DNA on to LB-Amp agar plates, colonies were picked

from the LB-Amp agar (no colonies on control.) and were grown in LB-Amp. Stocks were made by using 500 µl 50% sterilized glycerol and 500 µl culture and were stored at -80⁰ C.

2.17 Checking the protein expression of different mutants of GAPDH:

To check the protein expression, 3 individual colonies from each mutant GAPDH LB-Amp agar plate were selected and grown in 20 ml LB-Amp for 12-16 hours. The cells were pelleted by spinning at 5000 rpm at 4⁰ C for 10-15 minutes. For each 100 mg pellet 200 µl B-PER (bacterial protein extraction reagent) (PDT# 90084 thermo scientific) was added, cells were pipetted with B-PER until it is homogeneous, incubated for 15-20 minutes at 4⁰ C. Centrifuged at 15000 rpm for 5 minutes, our protein of interest is cytosolic protein and hence remains in supernatant. Supernatant was incubated with GST beads for 2 hours, GST beads (Pharmacia biotech# 17-0756-01) were washed with 1XPBS to remove unbound proteins which does not have GST, GST with GAPDH remains with the GST beads. The GST beads were then eluted with GSH (Sigma#G-4251) containing buffer and now GST with GAPDH was present in Buffer. Protein concentration was determined for this protein in buffer. The colony which expresses maximum protein of our interest is further used for purification in larger batches. Protein expression was determined by western blotting by using Anti-GST, since GAPDH which we use has a GST tag, so the band shows at around 60 KDa. Depending on

the intensity of the signal, colony that expresses optimum levels of protein of interest was selected.

2.18 Purification of myoglobin mutant:

Myoglobin mutant from sperm whale myoglobin was purified from a stock culture of E.coli strain ER2566 (NEB) and plasmid with pUC19 vector. 100 ml LB with 125 µg/ml ampicillin was inoculated with stock culture, grown overnight at 37⁰ C, shaking at 250 rpm. 4 liters terrific broth with 125 µg/ml ampicillin was divided into 8 flasks and into each flask 10 ml of overnight culture is inoculated and shaken at 37⁰ C for 1.5-2 hours and once stationary phase is reached which is measured at 600 nm by using spectrophotometer optical density should be around 0.8 to 1, the cells were spin down at 6500 rpm for 20 minutes at 4⁰ C. The pellets if not used immediately were kept frozen at -80⁰ C till use. The cell pellets were thawed and resuspended in 100ml lysis buffer which was made of 50 mM Tris HCl pH 8.0, 1 mM EDTA, 0.5 mM DTT, 0.5 mM DTT, 5 unit/ml DNase, 1 mg/ml lysozyme, PI#1 (5 mg/ml Aprotinin, 1 mg/ml Leupetin), PI#2 (1 mg/ml Pepstain A, 24 mg/ml Pefabloc SC in DMSO) and 1 µM PMSF and left on ice for 30 minutes. The cell suspension was sonicated at 36% amplitude for 35 seconds for 3 times. The sonicated cell suspension was centrifuged at 20000 rpm for 45 minutes at 4⁰ C to remove cell debris. The supernatant was brought to 60% ammonium sulfate saturation slowly; after 60% saturation was reached the solution was allowed to sit at 4⁰ C for 2 hours. The 60% saturated solution was centrifuged at

20000rpm for 30 minutes to remove precipitate, supernatant was then made to saturate with ammonium sulfate till 95%. The precipitate was collected by centrifugation at 20000 rpm at 4⁰ C for 30 minutes, stored at -80⁰ C till use. The precipitate from ammonium sulfate precipitation was resuspended in 30 ml 50 mM Tris HCl pH 8.0, 2 M Ammonium Sulfate and centrifuged again at 20000 rpm for 30 minutes at 4⁰ C to remove any pellet, supernatant was loaded onto phenyl sepharose column which was previously equilibrated with 50 mM Tris HCl pH 8.0, 2 M Ammonium Sulfate. Column was equilibrated with 3-4 column washes of 50 mM Tris HCl pH 8.0, 2 M Ammonium Sulfate. Elute myoglobin with 2 column volumes of 20:80 ratio of 50 mM Tris HCl pH 8.0, 2 M Ammonium Sulfate (20): 50 mM Tris HCl pH 8.0 (80). Collected fractions were dialyzed against 50 mM Tris HCl pH 8.0 with 1 buffer change. Dialyzed sample was passed through the DE-52 column which was equilibrated with 50 mM Tris HCl pH 8.0 and the flow through was collected. Myoglobin was concentrated to 1mM (17).

0.3 ml of the concentrated myoglobin solution was placed in 2-3 microcentrifuge tubes and place on ice. 100-300 µl of 0.1 M HCl was added to adjust pH to 2.3-2.5, 0.4 ml cold methyl ethyl ketone was immediately added to each of the tubes and the solution was left on ice for 1 minute and centrifuged for 2 minutes to separate 2 phases. The organic phase was removed and the process was done until there was no visible heme. The aliquots were combined and dialyzed against cold water for 4 to 6 hours and repeated for 3 to 4 times. The samples were lyophilized

and stored at -80°C . The samples were reconstituted with buffer and concentration was measured at 280nm by using extinction coefficient 15200 /M CM.

2.19 Purification of GAPDH:

500 ml of LB was inoculated with glycerol stock of GAPDH and 1 ul/ml of ampicillin was added, incubated at 37°C with shaking at 250 rpm for 14 to 16 hours. 50 ml of grown culture was transferred into 500 ml TB with 1ul/ml ampicillin and incubated at 37°C with shaking at 250 rpm until optical density reaches 0.8 to 1. The cultures were induced with 1 mM IPTG and the cultures were left shaking at 250 rpm for 48 hours at room temperature. Cells were spun down at 7000 rpm at 4°C for 30 minutes. Supernatant was discarded and the cell pellets were stored at -80°C until used. Lysis buffer was prepared using 100 ml PBS, 0.5 mM EDTA, 1 mg/ml lysozyme, 1 mM PMSF/DMSO, PI#1, PI#2. Pellet was thawed using 10 to 13 ml lysis buffer and gently suspended. Suspension was sonicated for 35 seconds at 36% amplitude by altered pulses for 7 seconds. This step was repeated for 3 to 4 times until the suspension become thinner and darker. The suspension was centrifuged at 18,000 rpm for 1 hour and the supernatant was collected, incubated with GST sepharose resin for 2 hours at 4°C . The GST sepharose resin was prepared by washing and equilibrating with 1XPBS. GST sepharose resin was spun down at 600 rpm for 3 minutes at 4°C and thick supernatant was discarded, as the desired protein was bound attached to GST sepharose resin. This step was repeated by adding 1XPBS and for 2 to 3 times, and

PBS with resin was added to column. For elution, buffer with 100 mM Tris-HCL pH 8, 10 mM GSH was prepared. Elution buffer was added to the column having the GST resin and agitated for 1-2 minutes and the flow through was collected. This step was repeated for 2 to 3 times to collect all the GST bound protein. The collected protein was concentrated using Amicon 30 K concentrator to 2-3 ml and washed with 1XPBS with 3 mM DTT and concentrated to 2 to 3 ml again. Thrombin (10 unit/mg 50 μ l) was added to this concentrated protein which cleaves the GST tag from protein, incubated at 37⁰ C for 2 hours, the protein with thrombin was incubated with GST resin at 4⁰ C for 2 hours to remove any protein which has GST tag, flow through was collected. The protein was then passed through Benzamidine FF resin (GE heath care#17-5123-10) which has been previously equilibrated with 50 mM Tris HCl and 500 mM NaCl, pH 8 to remove excess thrombin. The protein was concentrated to 2 to 3 ml by using Amicon 30 K concentrator, washed with 50 mM Tris pH 8 150 mM NaCl, 20% glycerol (Fisher bio reagents# BP229-4) for 2 to 3 times and was concentrated to 2 to 3 ml. To determine the purity SDS-PAGE was performed, our desired protein (GAPDH) has molecular weight of 37 KDa approximately (11).

2.20 SDS-PAGE:

SDS-PAGE was used to separate proteins by their molecular weight. The lower gel (separating gel) was made by using 5.6 ml 30% acrylamide (National diagnostics# EC-890), 5 ml 1.5 M Tris (Sigma# T1503-1 kg 99.9% pure), pH 8.8

with SDS (CAL Biochem# 428015), 9.1 ml water, 100 µl of 10% APS (Amresco#K833-100tabs), TEMED (Sigma# T9281-50ml) 20 µl, which indicates the separating gel is a 8.5%. The upper gel (stacking gel) was prepared using 1.3 ml 30% acrylamide, 2.5 ml 0.5 M Tris pH 6.8 with SDS, 6.1 ml water, 50 µl 10% APS, 20 µl TEMED. Samples were mixed with sample buffer which consists of glycerol, SDS, 2-mercaptoethanol (Sigma# M-3148), bromophenol blue and water, boiled for 5 minutes at 95⁰ C and were cooled down to room temperature. The protein samples were then loaded onto the gel. Non- reducing sample buffer which has no 2-mercaptoethanol was used for mass spectrometry experiments, where the protein samples were reduced during mass spectrometry sample digestion. The gel electrophoresis was performed at 90-100 V. Once the electrophoresis was finished, the gel was removed and stained with coomassie blue for 1 hour and destained using 30% methanol and 7% acetic acid overnight. The protein was identified by the blue bands and the molecular weight was determined using commercially available standards (Precision blue protein marker from Bio-Rad# 161-0373).

2.21 Western blotting technique:

It is a widely used technique to detect the specific protein or the protein of interest. This is also known as immunoblotting as it uses specific antibodies to detect the specific protein. After the gel electrophoresis was done, gel was washed with water for 10 minutes and equilibrated with semidry transfer buffer. The PVDF

membrane of size of gel was cut and activated with methanol, blotting sheet was equilibrated with semidry transfer buffer. The equilibrated blotting sheet was placed on transfer apparatus and then PVDF membrane, followed by gel and finally with one more equilibrated blotting sheet. Air bubbles should be avoided. Transfer was done for 40 minutes at 6 amperes current. The proteins were then transferred onto PVDF membrane. The blocking of PVDF membrane was done by using 5% non fat dry milk in PBS mixed with tween 20 overnight. The blocked PVDF membrane was incubated with primary antibody which was made by diluting antibody in 5% non fat milk for an hour at room temperature. The PVDF membrane was then washed with PBST for 3 times and incubated with secondary antibody for 1hour and then washed with PBST for 3 times and finally washed with PBS before adding substrate. ECL kit which is enhanced chemiluminescence was used for detecting secondary antibody which in turn gives signal about primary antibody and the primary antibody is specific for each protein. The detection solutions A and B were mixed in ratio of 40:1 on PVDF membrane after secondary antibody and washing steps, incubated for 5 minutes and after 5 minutes excess detection reagent was drained out and the PVDF membrane was placed on a cassette by placing it inside a wrap and the interface should be black. Expose Kodak scientific imaging film on the wrapped PVDF membrane in the dark. Film was developed. The signal was seen as dark band. The intensity of the signal is proportional to the amount of protein in the original sample. In biotin switch technique only one antibody was used which is biotin antibody which is

HRP conjugated and directly interacts with biotin. Here after blocking with milk wash PVDF with PBST and then finally with PBS followed by incubation with biotin antibody for 1.5 to 2 hours. Then ECL technique was used as previously described and Kodak scientific imaging film is used to detect the chemiluminescence signal which is due to substrate bound to HRP conjugated antibody which in turn indicates the specific protein.

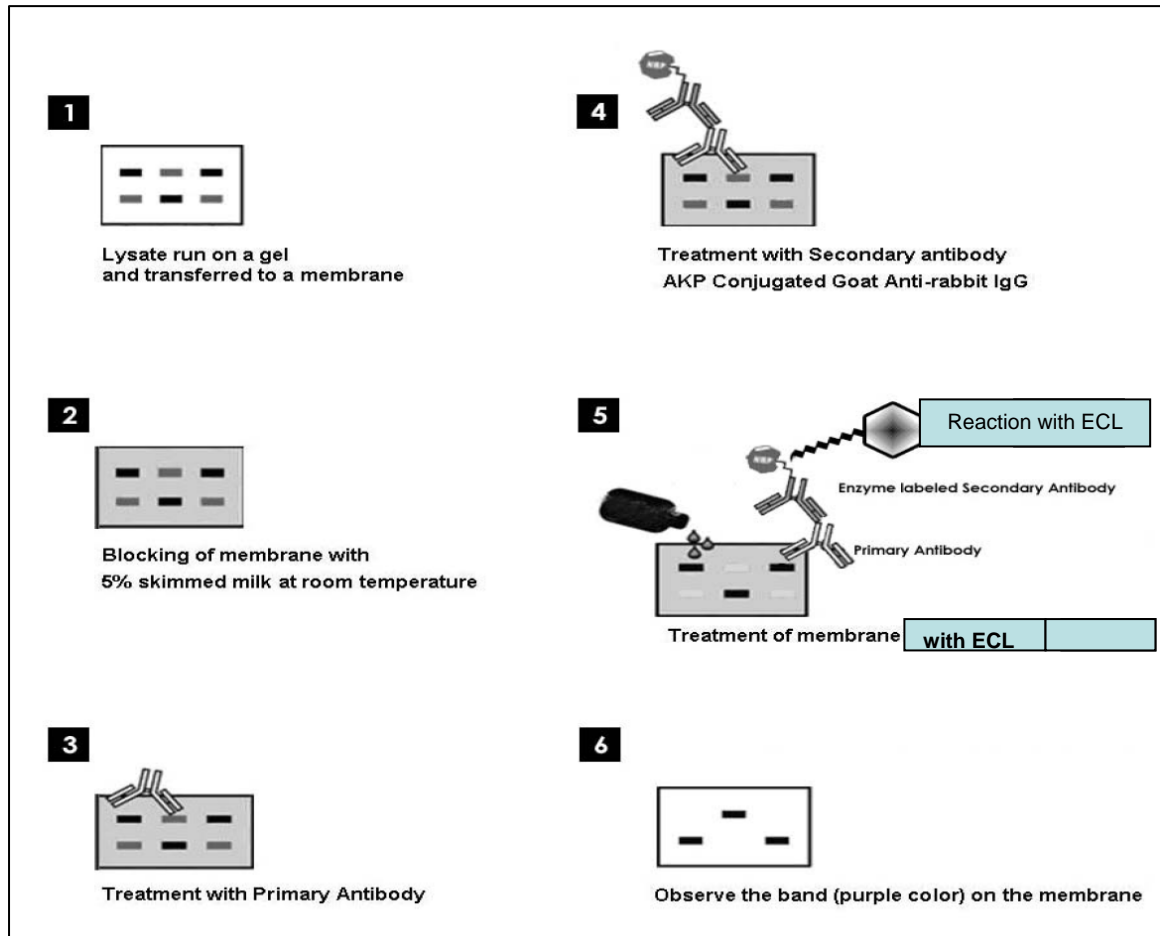


Fig.2.2. Schematic representation of Western blot technique.

2.22 Estimation of the extent of nitrosylation by SNOB reagent using western blot:

Stock concentration of 10.6 mM was prepared by dissolving 1.5 mg of SNOB reagent (Tocris# 3879) and SNOB control (Tocris# 3880) in 50 μ l of DMSO, then dissolved in 500 μ l PBS, stored at -20° C. The stocks were stable for a month in the dark at -20° C. Nitrosylation of the protein was carried out as previously described. 50 μ M of nitrosylated and non nitrosylated GAPDH was treated with 20 fold excess of SNOB reagent and control for 30 minutes at 60° C or 2-3 minutes exposing to high intensity light and excess SNOB was removed by passing through the PD-10 columns. The concentration of protein was determined and the same amount of protein was loaded into the gel with non-reducing sample buffer and gel electrophoresis was performed. After the gel was done the electrophoresed proteins were transferred onto PVDF membrane and immunoblotted using a biotin antibody. Mechanism of working of SNOB reagent was represented below.

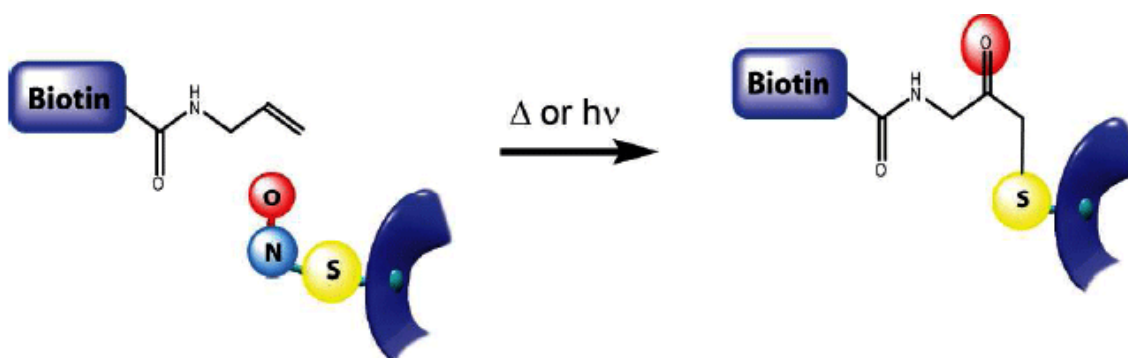


Fig.2.3. Reaction of the SNOB reagent with nitrosylated protein.

2.23 Sample preparation for mass spectrometry to determine the extent of nitrosylation on each individual cysteine:

Human and rabbit GAPDH were nitrosylated as previously described. 50 µm of 200 µl GAPDH were taken and 50 mm of 400 µl of NEM was added, incubated at room temperature for 1.5 to 2 hours. Excess NEM (Thermo scientific# 23030) was removed by acetone precipitations which was done by adding 20 volumes pre-chilled acetone to protein and incubated at -20⁰ C for 20 minutes, centrifuged at minimum of 2000 rpm for 10 minutes, precipitate was dissolved in 100 µl Hepes buffer with 1% SDS. 4 mM of 300 µl biotin HPDP (Pierce net# 21341) and 50 mM ascorbate solution was added to this dissolved protein. The samples were then mixed with non-reducing sample buffer and loaded onto gel and gel electrophoresis was performed. The staining was done using the gel code blue reagent, for overnight and then samples were subjected for mass spectrometric analysis.

Biotin HPDP was replaced with alkylating agent iodoacetamide (Acros organics# AC12227-0050), since we got very less biotinylation, when compared to nitrosylation with Saville's method. In iodoacetamide method after acetone precipitation, protein was dissolved in HENS buffer and 10 mM DTT for 100 µm protein, incubated for 2 to 3 hours. Iodoacetamide was added in excess which is 4 to 5 fold excess of DTT as DTT reacts with iodo acetamide and incubated for 2 hours. Proteins were mixed with sample buffer which is non-reducing with no

boiling and loaded onto gel and gel electrophoresis was performed. The gel was stained with gel code blue (Thermo#24592) and subjected to mass spectrometry.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Characterization of Heme transfer reactions using GAPDH-heme to apomyoglobin as a model:

My project focuses on kinetics of heme dissociation from the GAPDH-heme complex which is measured by the rate of heme binding to apomyoglobin. This was measured by the absorbance changes occurring at 410nm with time for proto-heme and at 398nm for meso-heme.

The typical spectrum of the GAPDH-heme complex looks like below after passing the GAPDH-heme complex through PD-10 columns where free heme was removed and only heme bound to GAPDH was eluted. Typical ratio of heme bound to GAPDH is 0.5 heme to 1 monomer GAPDH.

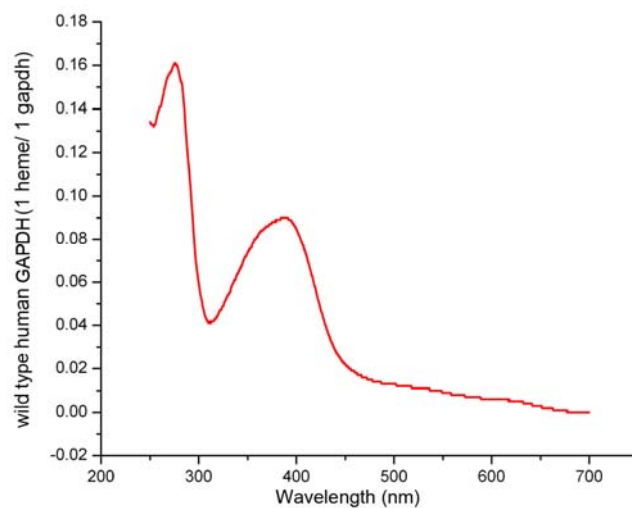


Fig.3.1. UV-Vis spectrum of heme bound GAPDH. The near 400nm peak represents heme bound to GAPDH peak and the wavelength maximum at 280nm represents GAPDH peak.

A typical time course for the transfer of heme from GAPDH to apomyoglobin was shown in Fig.3. 2. The rate difference was monitored by spectral changes against time.

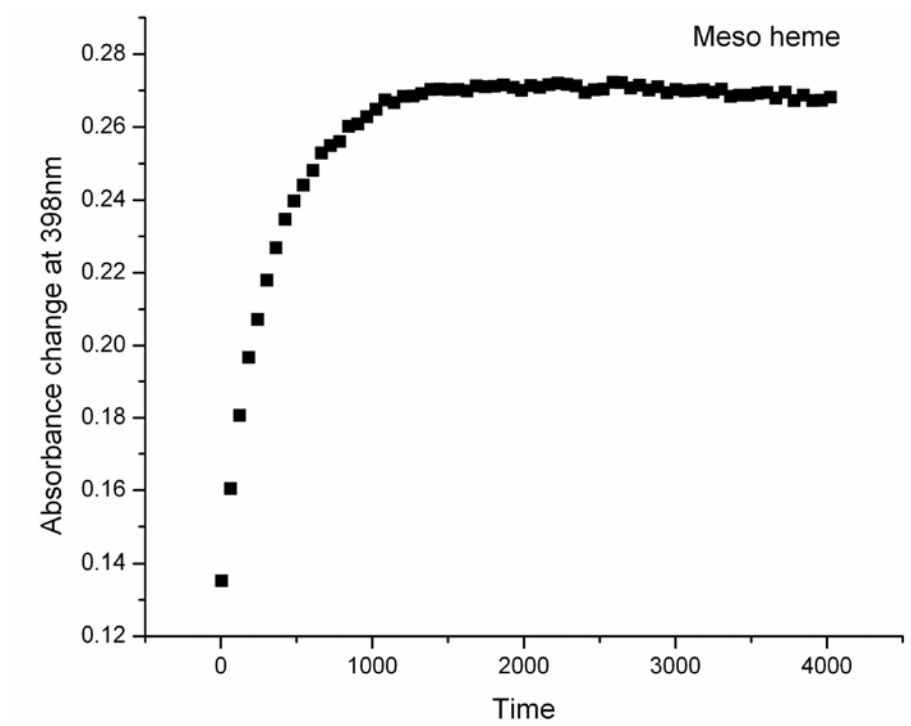


Fig.3.2.1 Typical curve for meso heme transfer from GAPDH-heme complex to apomyoglobin. The monitored wavelength is 398nm for meso-heme.

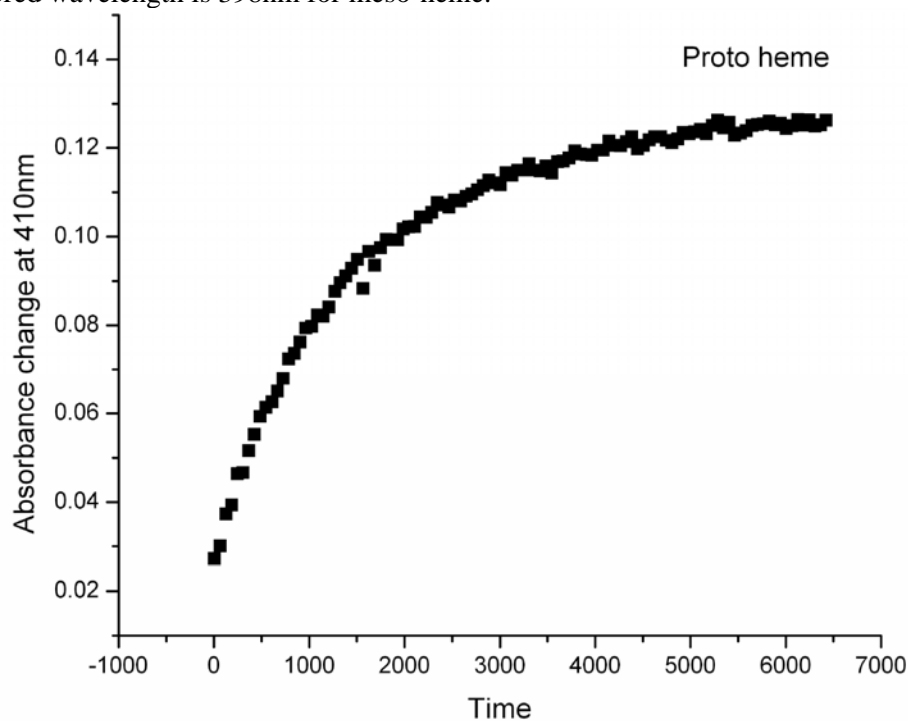


Fig.3.2.2 Typical curve for proto heme transfer from GAPDH-heme complex to apomyoglobin. The monitored wavelength is 411nm for proto-heme

The rates of heme transfer were different for 2 heme types which were considered here. The 2 types of heme which are considered here are meso and proto heme. The rate of heme delivery was 2 times faster for meso heme compared to that of proto heme which is shown in the Fig.3.3. Meso heme differs from proto heme in the side chain.

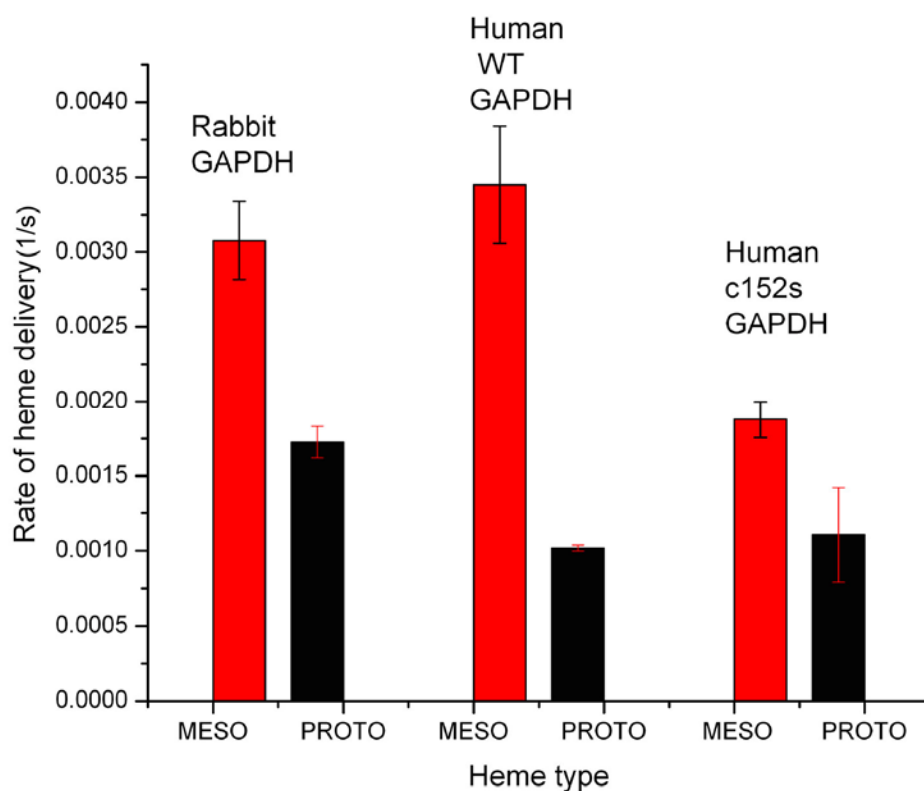


Fig.3.3. In the above graph red column represents meso heme which shows that it has faster rate of heme delivery compared to that of proto heme which is represented by black column.

The rate of heme transfer from GAPDH-meso heme complex is faster to apomyoglobin, compared to the rate of heme transfer from GAPDH-proto heme

complex. The reason may be that, meso heme is less strongly bound to GAPDH, when compared to binding of proto heme to GAPDH.

We focused on factors affecting the rate of heme transfer.

Effect of GAPDH-Heme complex concentration on rate of heme transfer to apomyoglobin:

We examined if GAPDH-heme complex concentration has any effect on heme uptake by apomyoglobin by maintaining same ratio of complex to apomyoglobin ratio. GAPDH-heme complex was used in different concentrations in each well and apomyoglobin added was 3 fold excess to that of heme concentration. Changing the concentration of complex has no effect on rate of heme transfer to apomyoglobin, as shown in Fig.3.4.

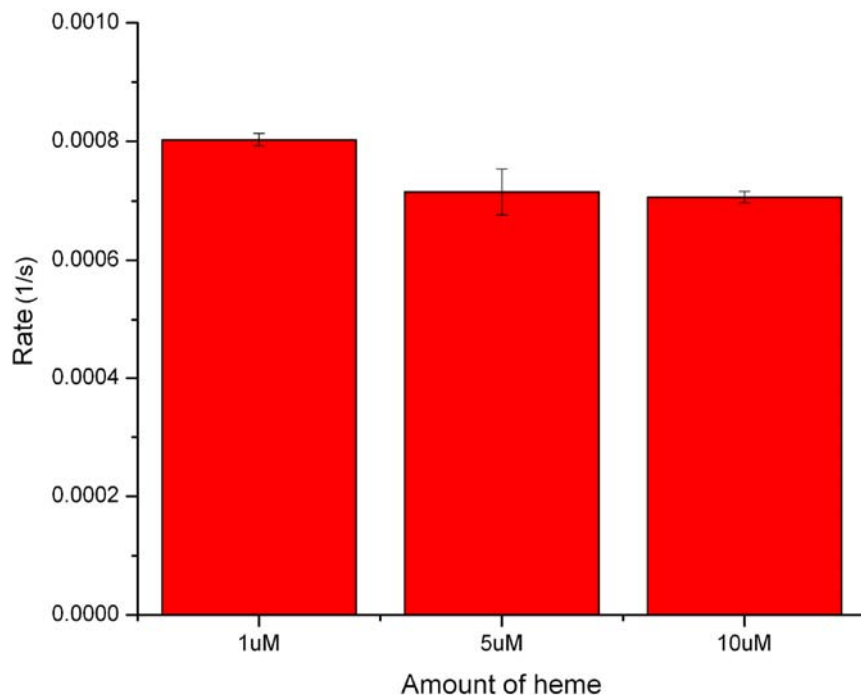


Fig.3.4. Comparing different amount of GAPDH-heme complex to same fold of apomyoglobin. As the results show it has no substantial effect on rate of heme transfer.

Effect of heme: GAPDH ratio on the rate of heme transfer to apomyoglobin:

We examined if the amount of heme bound to GAPDH influences the rate of heme transfer to apomyoglobin. GAPDH-heme complexes were prepared considering 100uM GAPDH as constant and increasing heme concentrations from 100uM, 200uM, 400uM, 800uM and passed them through PD-10 gel filtration columns to remove free heme. Same protein concentration, different heme concentration and maintained same apomyoglobin: heme ratio was maintained in each well. The results from this experiment suggest that the rate of heme uptake by apomyoglobin is independent of amount of heme bound to GAPDH.

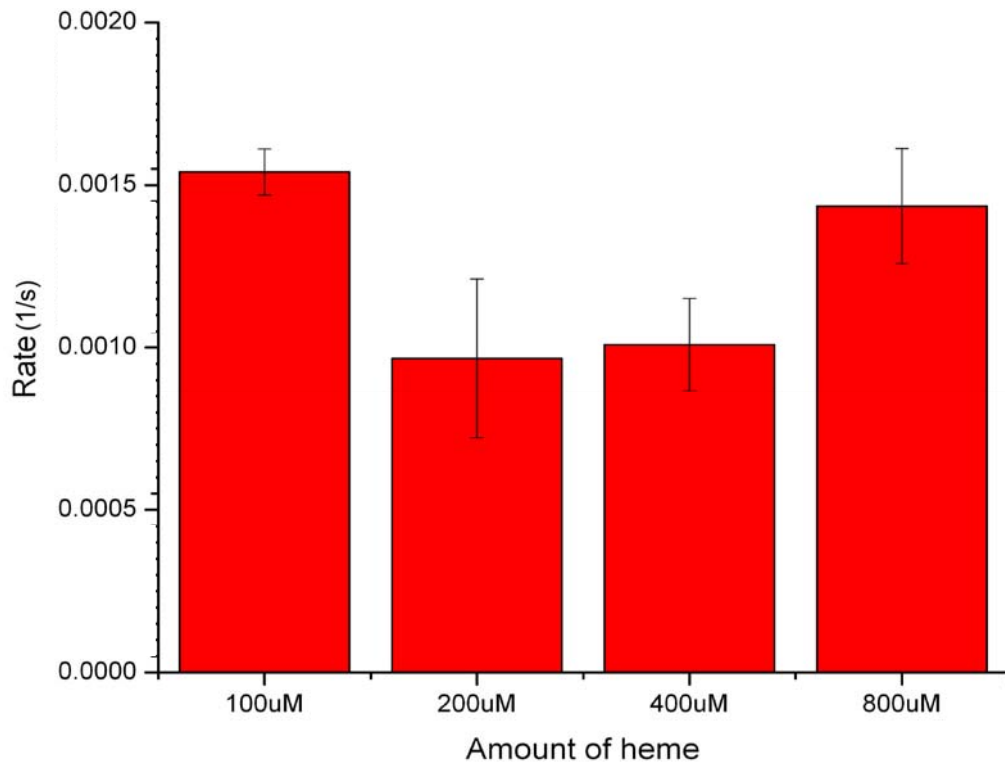


Fig.3.5. Comparison of rates of heme transfer when different amount of Heme placed into each well maintaining GAPDH same. The results show that there was not much substantial difference in rate of heme transfer.

3 complexes were made with same GAPDH concentration and with different heme concentrations. Same amount of heme and apomyoglobin were loaded into each individual well. Here the variable was GAPDH. The effect seems to be increasing as GAPDH is decreasing but it's less than 20% which is within the experimental error range.

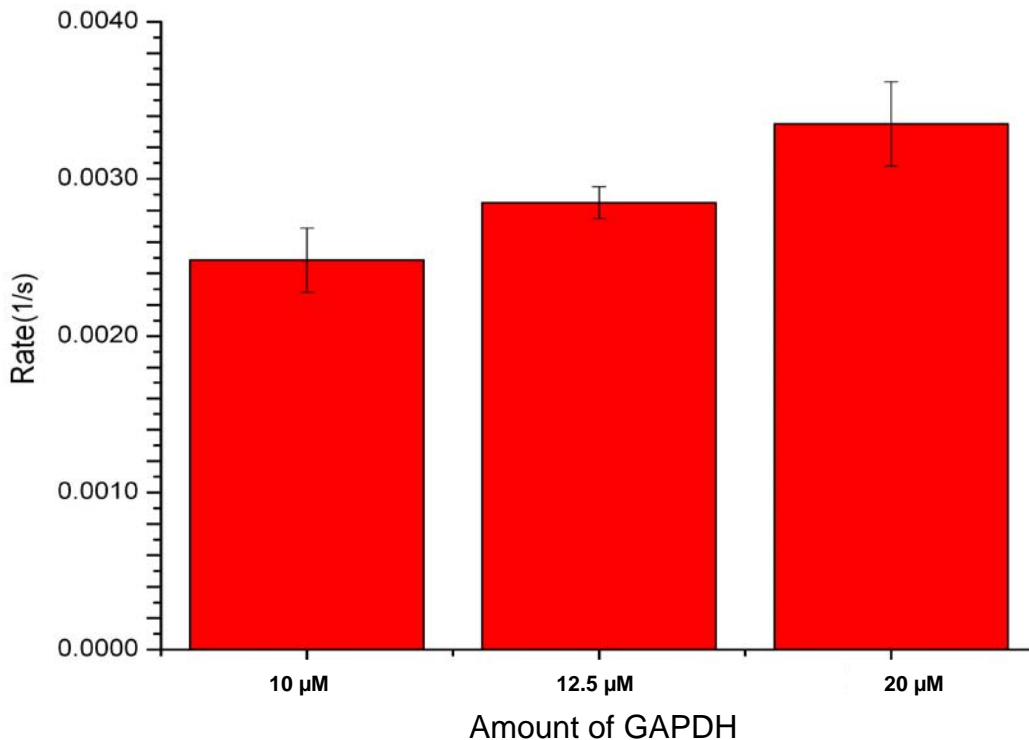


Fig.3.6. Comparison of rate of heme transfer when different amount of GAPDH is placed into well by maintaining same amount of heme into final well. The result shows that the effect is not substantial, although it shows that rate is increasing with lowering amount of GAPDH.

Effect of NO on the rate of heme transfer from GAPDH to apomyoglobin:

We examined if any external factors has an affect on rate of heme uptake by apomyoglobin. We here focused on NO as it has many physiological roles, since specific nitrosylation of GAPDH at cysteine 152 was shown to block cellular

heme insertion into iNOS (9). The first experiment which was considered was adding NO donor GSNO to GAPDH-heme complex incubating less than 5 minutes before adding apomyoglobin. As shown below here there is no substantial effect of NO on rate of heme delivery.

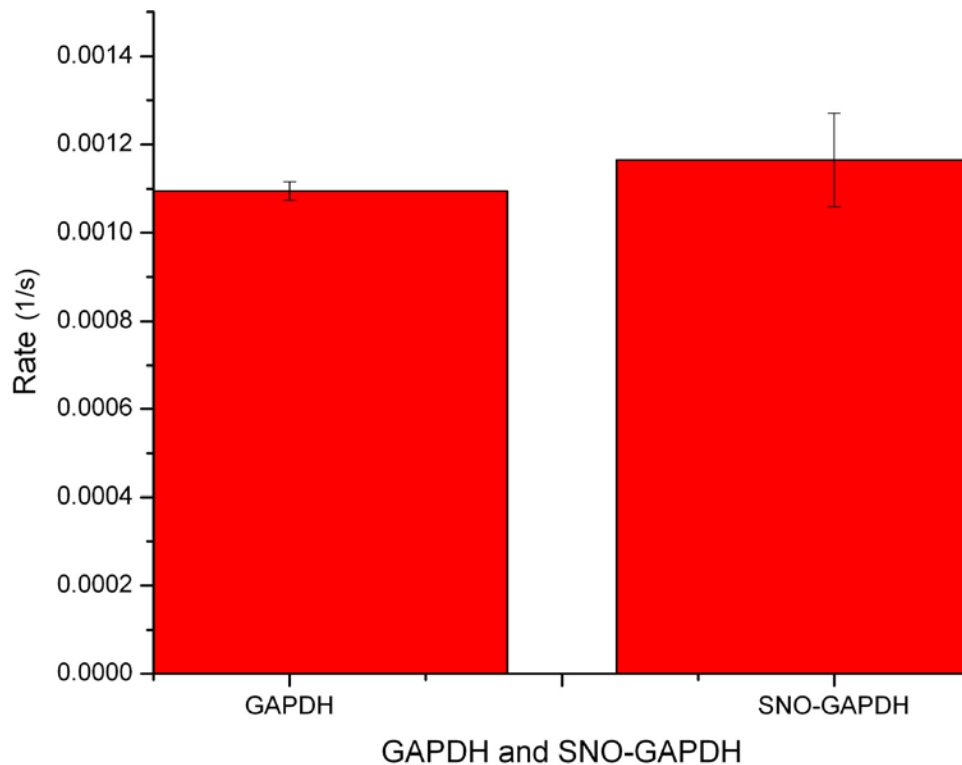


Fig.3.7. Comparison of rate of heme transfer from GAPDH-heme complex to that of GAPDH-heme complex with nitrosylation donor added to it before adding apomyoglobin. . The result shows that S-nitrosylation of GAPDH has no effect on the rate of heme transfer.

Effect of Hsp90 and cell lysate proteins on rate of heme transfer from GAPDH-heme complex to apomyoglobin:

We examined if Hsp90 has an affect on rate of heme transfer, which is a chaperone and is believed to have an involvement in heme insertion into a number

of proteins (8). Cell lysate fractions of human macrophages were prepared, rate of heme transfer to apomyoglobin from GAPDH-heme complex were compared to that of GAPDH-heme complex mixed with macrophage cell lysate and Hsp90. The results suggest that Hsp90, cell lysate proteins does not affect the rate of heme transfer. We maintained same GAPDH-heme complex to apomyoglobin ratio.

contents	value 1	value 2	average
a.) Apomyoglobin+ complex	8.999×10^{-4}	1.02×10^{-3}	9.599×10^{-4}
b.) Apo+ complex + cell lysate mix	1.29×10^{-3}	1.13×10^{-3}	1.21×10^{-3}
c.) Apo+ complex+ 50k and more	1.23×10^{-3}	1.41×10^{-3}	1.32×10^{-3}
d.) Apo+ complex+ 50k and less	1.02×10^{-3}	7.924×10^{-4}	9.062×10^{-4}
e.) Apo+ complex+ cell lysate mix+ HSP90	1.60×10^{-3}	1.02×10^{-3}	1.31×10^{-3}
f.) Apo+ complex+ HSP90	1.48×10^{-3}	1.10×10^{-3}	1.29×10^{-3}
g.) Apo+ complex+ 50k and more+ HSP90	1.16×10^{-3}	1.19×10^{-3}	1.175×10^{-3}
h.) Apo+ complex+ 50k and less+ HSP90	1.06×10^{-3}		1.06×10^{-3}

Table 3.1. The K_{off} values for each different combination were represented in the table 3.1.

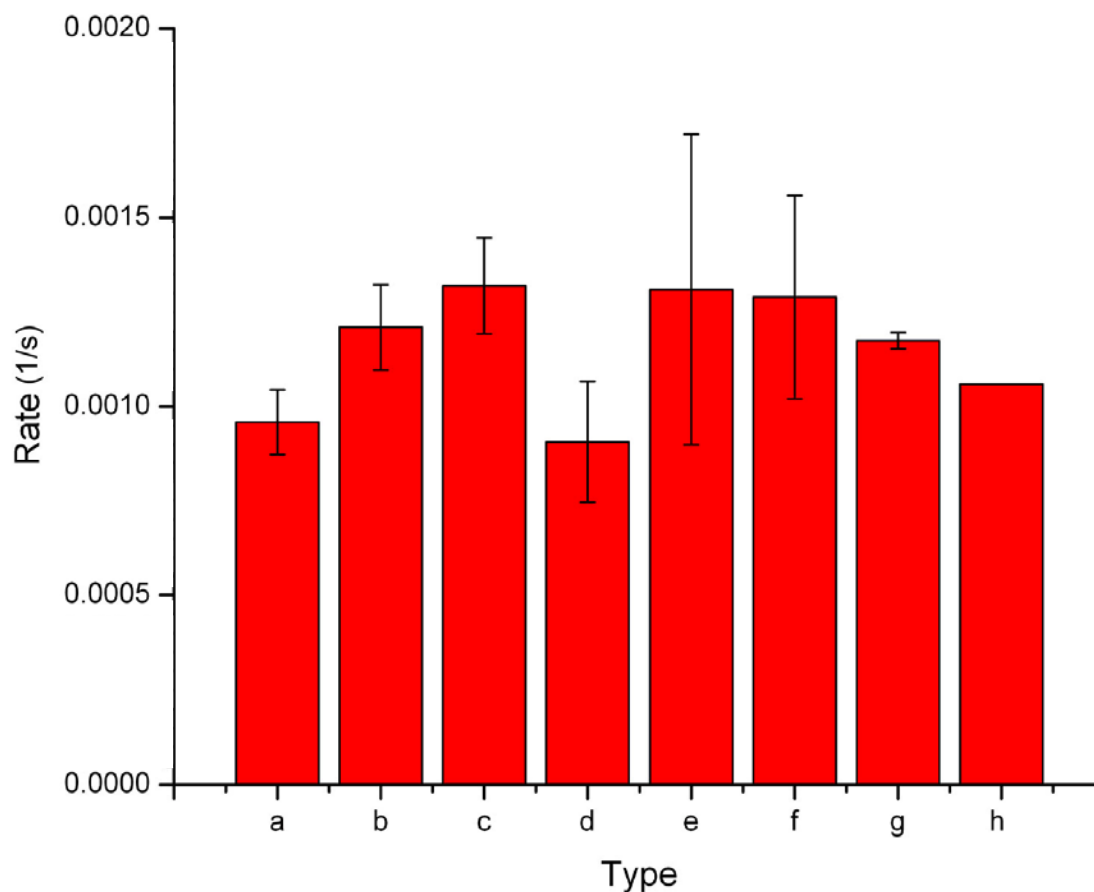


Fig.3 8. The above figure shows the effect of Hsp90 and macrophage cell lysate on heme transfer of GAPDH-heme complex. It shows very slight increase in rate of heme transfer when Hsp90 and also with macrophage cell lysate of 50kda and high compared to GAPDH-heme complex.

Effect of different doses of cell lysate on rate of heme transfer from GAPDH-heme complex to apomyoglobin:

We examined dose response curve of cell lysate with maintaining same concentration of GAPDH-heme complex and apomyoglobin and the only variable is cell lysate protein concentration. No substantial change was observed on the rates of heme transfer with varying concentrations of cell lysate.

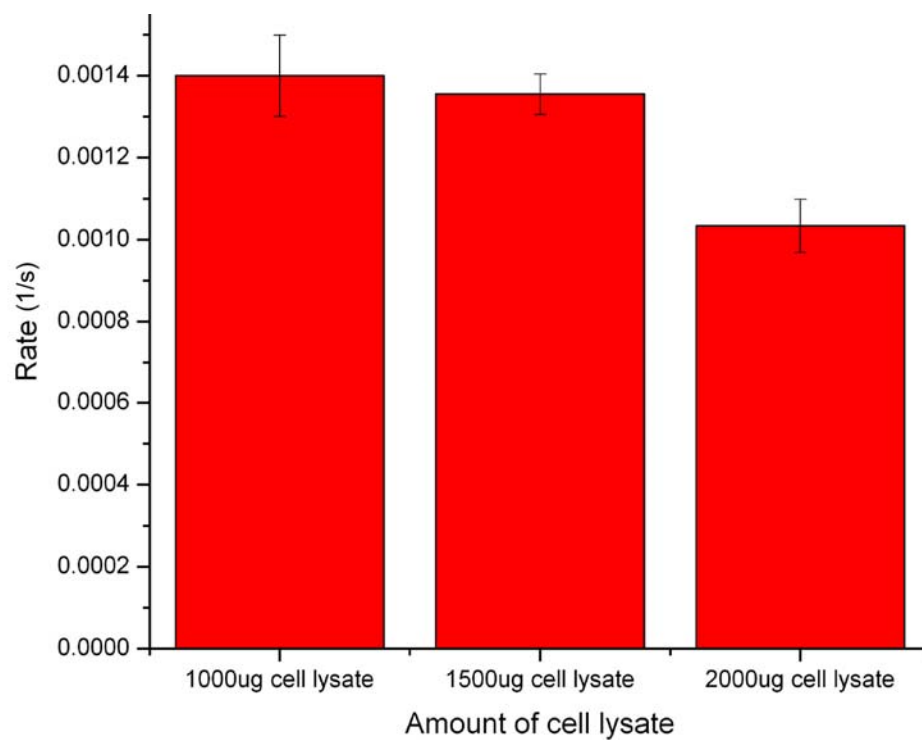


Fig.3.9. Comparison of rate of heme transfer when different concentration of macrophage cell lysate added to GAPDH-heme complex. The result shows that there was no substantial effect on rate with amount of cell lysate.

3.2 Tryptophan quenching:

This is used to check the binding between 2 proteins or 2 ligands. Here we tested the binding between different GAPDH with heme. Fig.3.10 shows a representative titration quenching experiment with different doses of heme. The result shows that there was strong binding between rabbit GAPDH and heme when compared with human GAPDH. The mutants show that there was not much difference in amount or strength of binding compared to human wild type. The mutants which we considered here were cysteine 152 and lysine 227 which were available in lab.

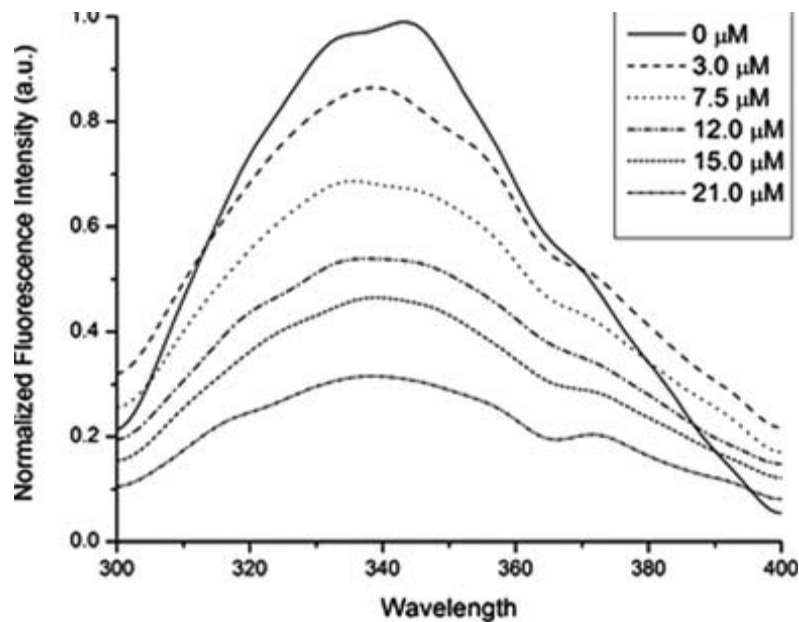


Fig.3.10. Above graph shows the quenching of fluorescence when adding increasing amounts of heme to GAPDH.

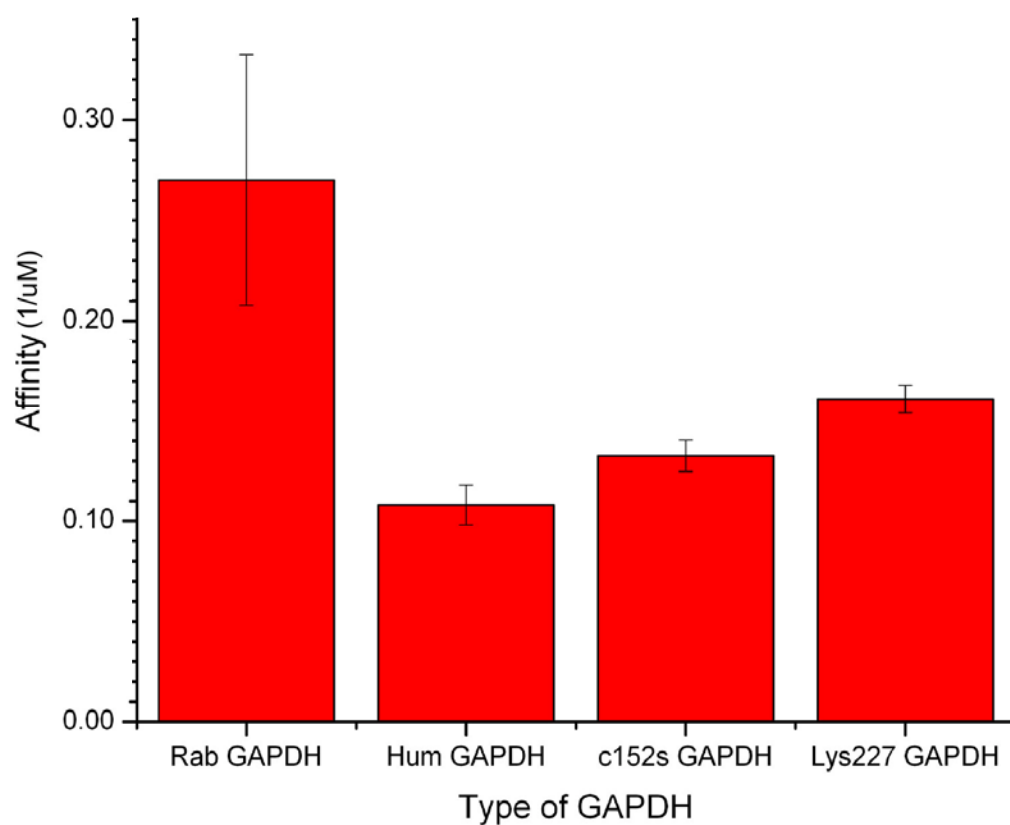


Fig.3.11. Above graph shows that rabbit GAPDH has more affinity towards heme compared to human GAPDH and there is no substantial difference between different mutants of human GAPDH although lysine 227 mutants appears to have slightly more competent binding.

3.3 S-Nitrosylation of GAPDH, its effect on activity and rate of heme delivery:

Previous work from our lab (9) has shown that by nitrosylating GAPDH the heme binding capacity of GAPDH was reduced. Based on this finding, I set out to investigate the effect of S-nitrosylation on the ability of GAPDH to transfer its heme into apomyoglobin. GSNO was used as NO donor to nitrosylate the GAPDH. The amount of nitrosylation is determined by the Saville's method which uses the Griess reagents. The activity is also used to check the nitrosylation of cysteine 152 as it is the active residue in GAPDH. GAPDH was nitrosylated as per the published protocol and the activity assay was performed before and after nitrosylation and also after incubation with heme. We also determined the rate of heme transfer to check the effect of nitrosylation on heme transfer of GAPDH. The results are shown in Fig.3.12.

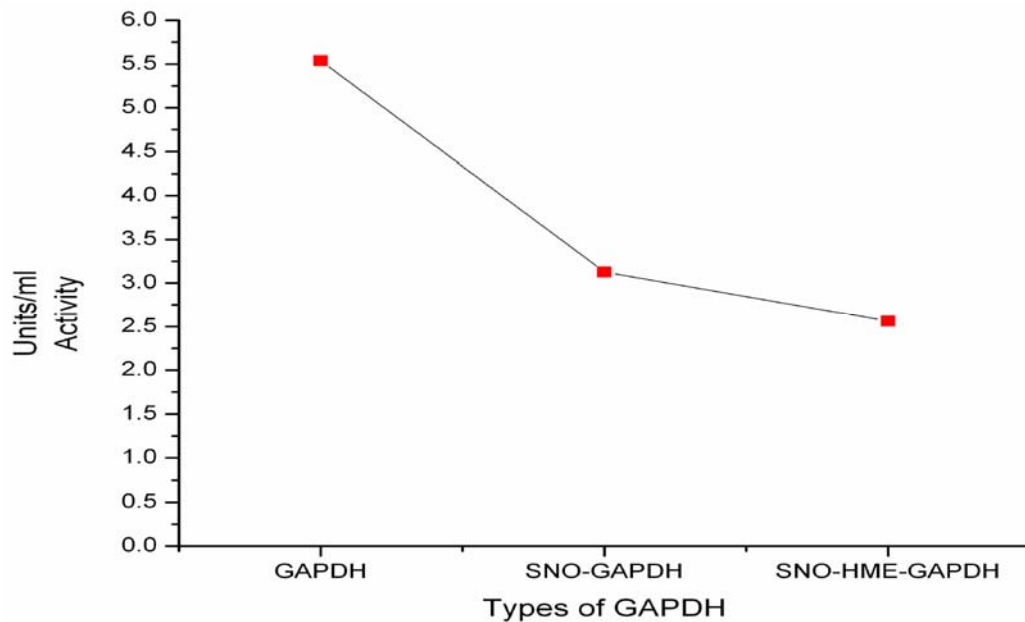


Fig.3.12. The graph shows that there was decrease in activity as protein gets nitrosylated. The heme does not have any significant decrease in activity.

As shown in the above graph there was a decrease in GAPDH activity upon nitrosylation, addition of heme had no effect on GAPDH activity.

Then, as described above, we measured the rates of heme transfer under these conditions. Similar rates were obtained for nitrosylated and non-nitrosylated proteins. . The extent of nitrosylation of our GAPDH preparation is determined by Saville's method. The level of nitrosylation under these conditions was very less (~15%). Dose response curve was performed to increase the extent of nitrosylation. No improvement in the extent of nitrosylation was obtained.

3.4 Nitrosylation and factors affecting the nitrosylation:

Physiological NO donor Cys-NO was used, which was prepared in laboratory by mixing sodium nitrite and L-cysteine with DTPA as described in method. We used bovine serum albumin to check the amount of nitrosylation as BSA was less expensive and easily available. Range of concentration of Cys-NO 10, 20, 30 and up to 150 fold was used with respect to protein concentration, low levels of nitrosylation (~30%) was obtained. Freshly prepared BSA and frozen BSA was treated with 100-fold excess DTT to check if any disulphide bonds present are interfering with extent of nitrosylation and excess DTT was removed by PD-10 columns. There was dramatic increase in the levels of nitrosylation by reducing BSA with DTT both in fresh as well as frozen samples. A similar finding was reported previously (33).

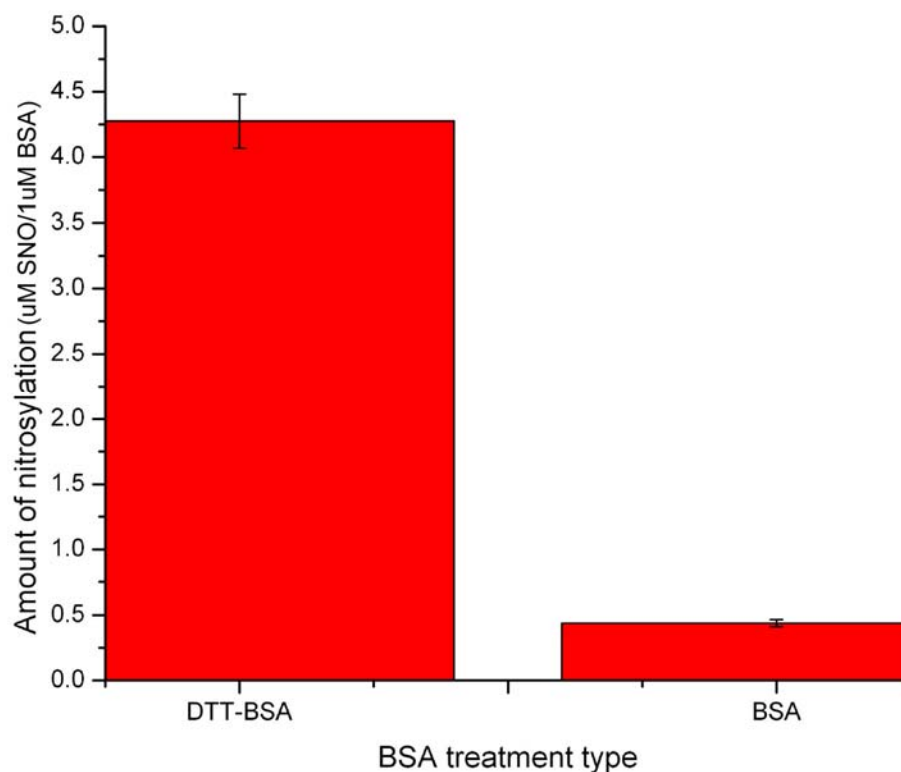


Fig.3.13. The difference in amount of nitrosylation of normal BSA and DTT treated BSA. The result shows that it has a substantial difference in amount of nitrosylation which indicates that the presence of disulphide bonds was interfering with the process of nitrosylation.

GAPDH was reduced with 100 fold excess DTT to check if any disulphide bonds are interfering with extent of nitrosylation. DTT treated and untreated GAPDH shows same amount of nitrosylation after nitrosylating with Cys-NO. The result suggests that there was no effect of DTT indicating that there were no disulphide bonds and dose 20 fold of nitrosylation donor shows having better nitrosylation.

We examined other factors which may have effect on nitrosylation of GAPDH like dose of NO donor and presence of reducing agents (example: DTT). Nitrosylation is light sensitive. Range of concentrations 10, 20, 50, 200 fold Cys-NO to that of

GAPDH was considered. The result suggests that there were no disulphide bonds which are interfering with nitrosylation and nitrosylation is not dose dependent.

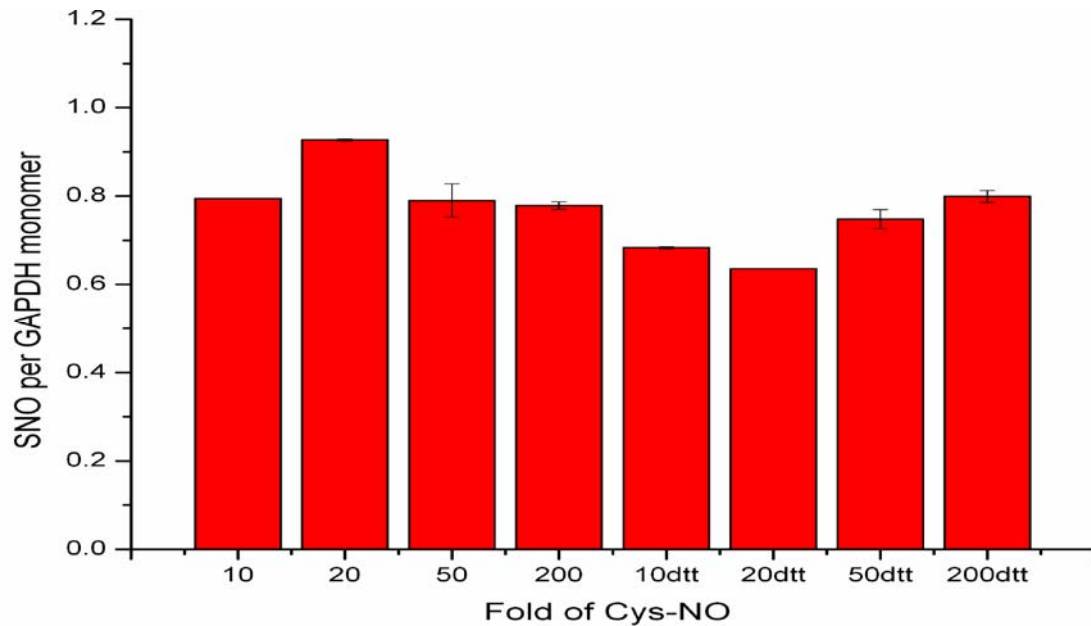


Fig.3.14. The amount of nitrosylation happening with increasing amount of nitrosylation donor and comparison with and with out adding DTT. The result suggests that there is no substantial difference on extent of nitrosylation.

We focused on dose response curve of different GAPDH to understand the affect of amount of nitrosylation donor on extent of nitrosylation on human recombinant wild-type GAPDH, c152s mutant human GAPDH and rabbit muscle GAPDH.

3.5 Dose response curves for S-nitrosylation of different GAPDH variants:

Rabbit GAPDH:

In case of rabbit GAPDH, the result shows no effect of dose of nitrosylation donor on extent of nitrosylation (Fig.3.15). The fold of Cys-NO which was considered, was 1, 2, 3, 5, and 10, 20 and 50-fold to that of protein concentration

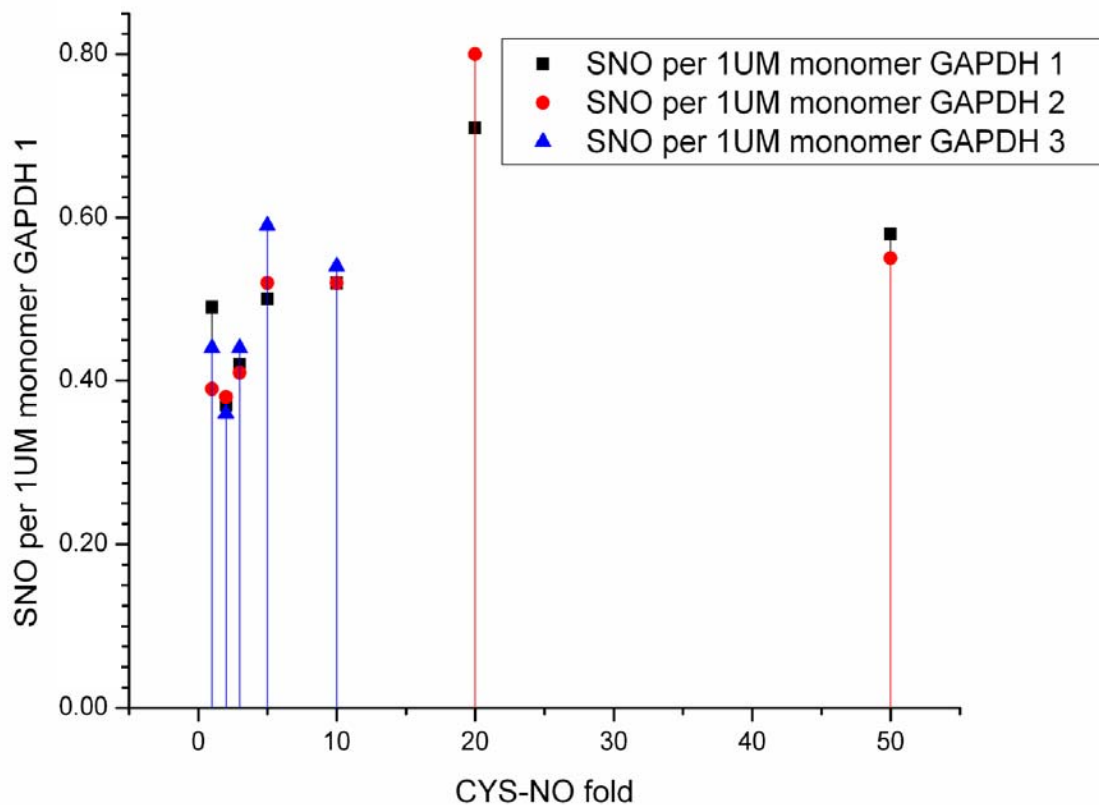


Fig.3.15. Comparing different doses of nitrosylation donor with same amount of rabbit muscle GAPDH. The results show that there was no substantial effect on the extent of nitrosylation though slightly higher nitrosylation was observed at 20 fold.

Human GAPDH:

In the case of human GAPDH the extent of nitrosylation is directly proportional to Cys-NO fold (Fig.3.15). The amount of nitrosylation was increased with amount of Cys-NO used. The fold of Cys-NO which were considered, were 1, 2, 3, 5, 10, 20 and 50 fold to that of protein concentration.

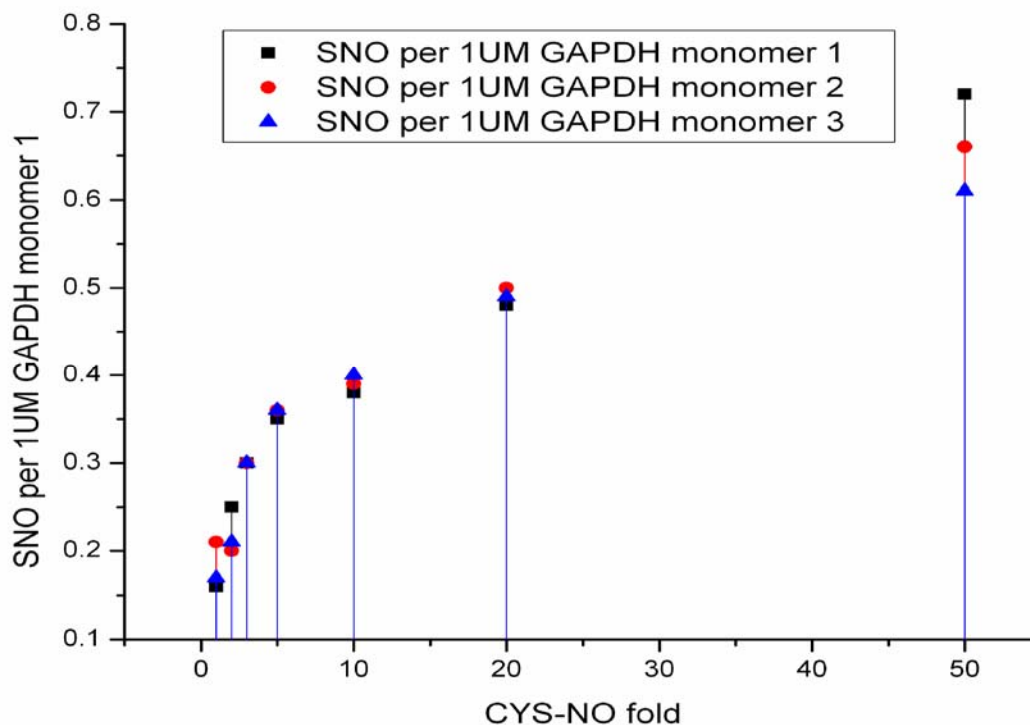


Fig.3.16. Comparing different doses of nitrosylation donor with same amount of Human GAPDH. The result shows that the nitrosylation is increasing with the amount of nitrosylation donor which shows that it has maximum effect at 50fold.

C152s GAPDH:

In the case of human C152S mutant GAPDH the extent of nitrosylation was directly proportional to Cys-NO fold. Surprisingly, we found that the extent of nitrosylation was similar to human wild-type GAPDH, *in vivo* experiments from our lab suggests that nitrosylation occurs only at cysteine 152, here with out cysteine 152 similar extent of nitrosylation was observed. Possibilities include that S-nitrosylation is specific *in vivo*, which was not observed *in vitro*. Protein DNA sequencing was performed to confirm the presence of mutation. Wild type has the activity and C152S has none, as expected.

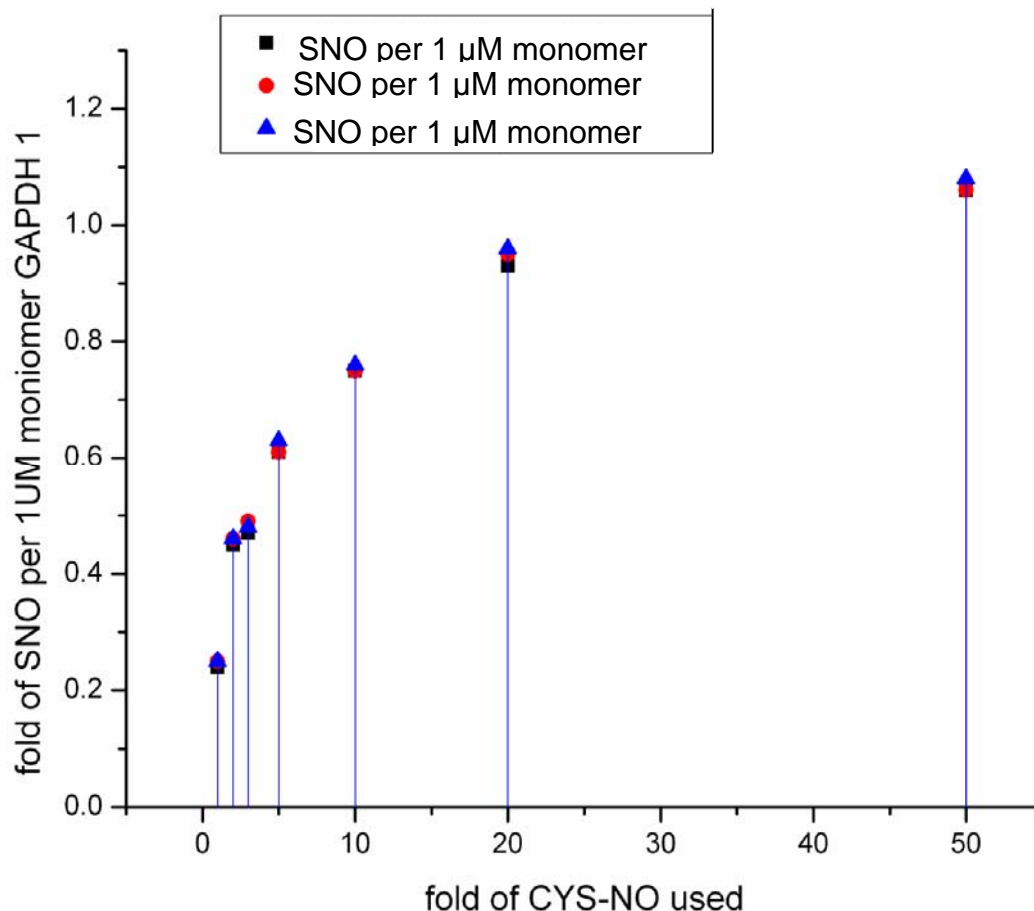


Fig.3.17. Comparing different doses of nitrosylation donor with same amount of Human c152s GAPDH, the result shows that the nitrosylation is increasing with the amount of nitrosylation donor which shows that it has maximum effect at 50fold.

3.6 Stability of nitrosylated GAPDH:

We examined the stability of nitrosylated GAPDH with time and the effect of heme on stability of nitrosylated GAPDH. GAPDH was nitrosylated using 50-fold excess Cys-NO. The stability tests were conducted at 0, 2, 6, 18, 48, 72, 120 hours. Stability test for nitrosylated-GAPDH was performed at two conditions: room temperature and 4°C. We took both rabbit GAPDH and human GAPDH to compare the results.

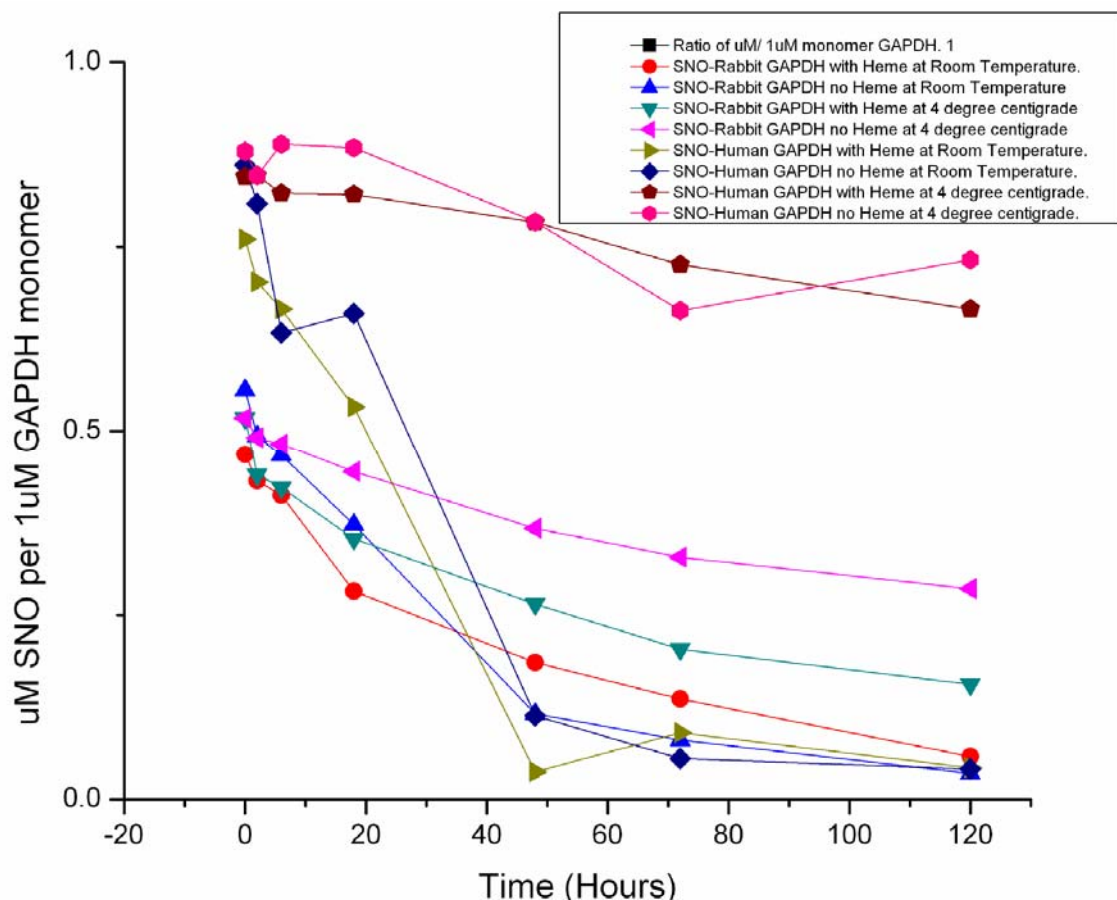


Fig.3.18. The amount of nitrosylation at different time points at room temperature and 4 degree centigrade with and without heme. As the time progresses the amount of nitrosylation decreases which is drastic at room temperature and less at 4 degree centigrade. The proteins which were considered here are human and rabbit GAPDH and the nitrosylation donor used was 50 fold.

From the above graph (Fig.3.18) it is observed that levels of nitrosylation decreased with time and at room temperature the nitrosylation drops down drastically. Human GAPDH at 4°C is stable for up to 5 days the presence of heme does not have any effect on the stability of rabbit and human nitrosylated GAPDH. From the above experiment one thing that is confirmed is that temperature plays major role in the stability of nitrosylated GAPDH besides light.

Effect of pH on S-nitrosylation of GAPDH:

The other factor which I considered was the pH as we are utilizing a dose of 50-fold excess Cys-NO to that of protein concentration. Here the pH's which I considered was near physiological pH's which are 6, 7, and 8. The effect was found to be as the pH increases the nitrosylation of GAPDH increases in case of rabbit and in case of human it is shown to have increased yields of nitrosylation at acidic and alkaline and remain less at neutral pH at 7. Here 3 buffers of different pH were made and GAPDH solutions were made with those buffers. Then 50fold Cys-NO was added and rest is as usual and PD-10 columns were also equilibrated with respective buffers. Altogether, these results suggest that human and rabbit GAPDH present differences in their ability to undergo S-nitrosylation at different pH's, which may be due to the extra exposed cysteine present at 282 position in rabbit GAPDH.

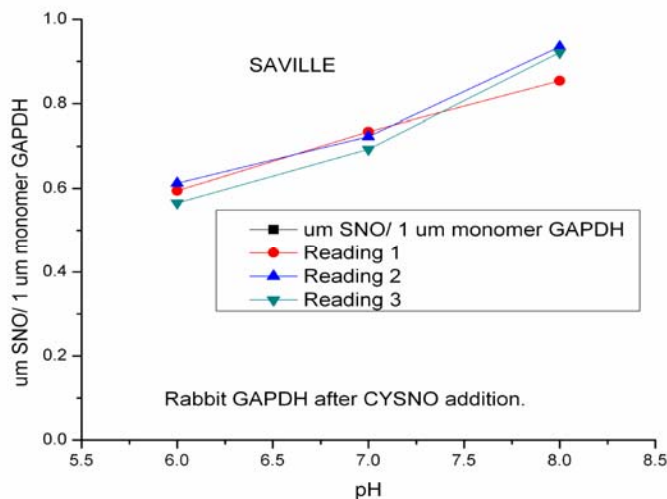


Fig.3.19. Comparing the amount of nitrosylation on rabbit muscle GAPDH which are nitrosylated at 3 different pH 6, 7 and 8. The result shows that the degree of nitrosylation slightly increases with increase in pH which shows maximum nitrosylation at pH 8.

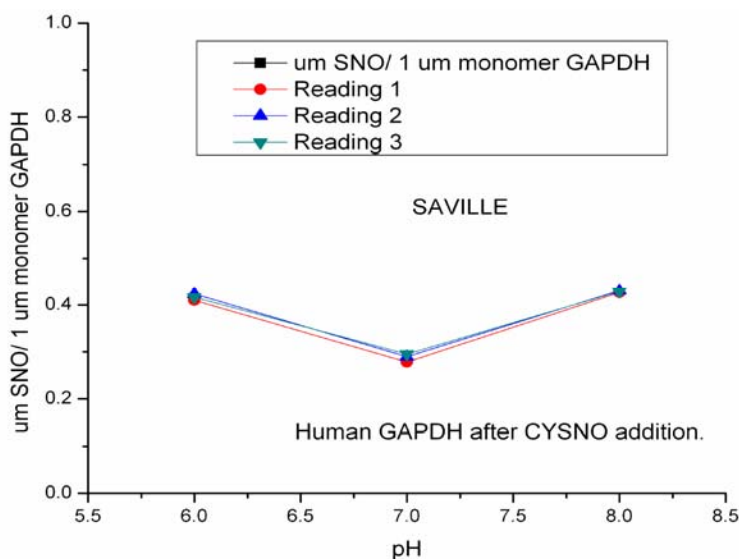


Fig.3.20. Comparing the amount of nitrosylation on human GAPDH which were nitrosylated at 3 different pH's 6, 7 and 8. The result shows that the degree of nitrosylation is independent of pH.

Effect of oxygen on the efficiency of S-nitrosylation of GAPDH:

We examined the affect of oxygen on extent of nitrosylation (As oxygen can cause free thiol oxidation). Nitrosylation was performed in anaerobic chamber to determine the affect of oxygen on extent of nitrosylation of GAPDH. All the buffers and reagents and also PD-10 columns were degassed by using nitrogen prior to experiment. We considered 3 different pH's 6, 7 and 8. The result from Fig.3.21, which when compared to Fig.3.20 suggests that there were no oxidized cysteines in human GAPDH. A comparison of aerobic and anaerobic conditions shows no difference in the yield of nitrosylation.

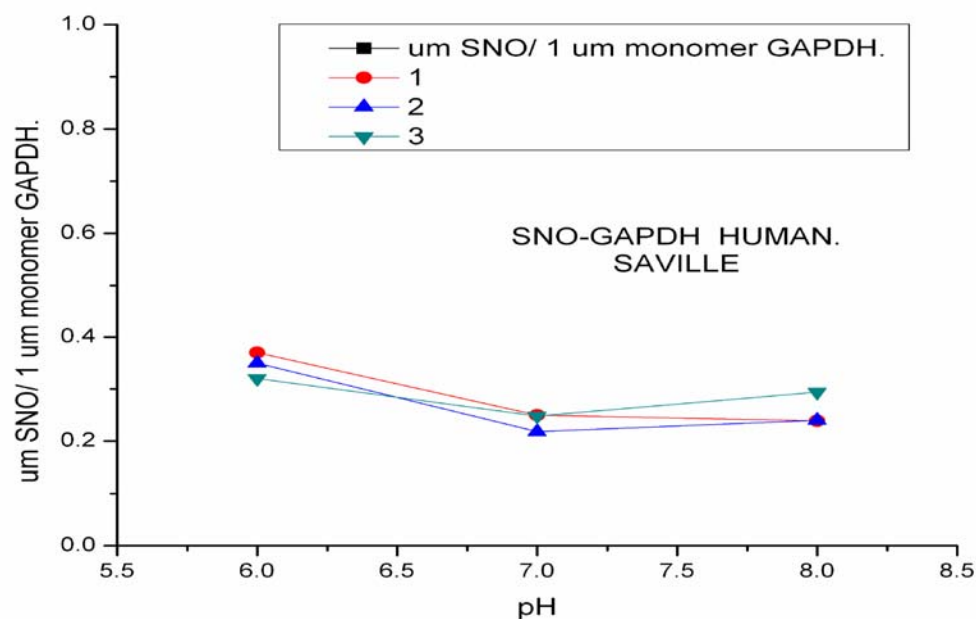


Fig.3.21. Comparing the amount of nitrosylation on human GAPDH in absence of oxygen which were nitrosylated at 3 different pH 6, 7 and 8. The result shows that the amount of nitrosylation is independent of pH and oxygen.

Finally, we considered whether or not the use of HEPES buffer was most appropriate for these experiments. Although not previously reported we suspected that tertiary amines may interfere with nitrosylation by reacting with Cys-NO. We also checked PD-10 column effectiveness in terms of removing unreacted Cys-NO. So here we incubated HEPES with 50ul which generally is 50fold or more when considered protein for 30-45 minutes and passed it through the PD-10 columns and found negligible amount of NO. By this experiment we confirmed, HEPES buffer has no effect on S-nitrosylation and PD-10 gel filtration columns blocks unreacted NO donor.

3.7 Estimation of the extent of s-nitrosylation on each individual cysteine by mass spectrometry:

Extent of nitrosylation on each individual cysteine residue was identified by mass spectrometry as we have seen C152S mutant GAPDH was getting nitrosylated, which was different from *in vivo* data. SNOB technology was used as this is a single step reaction which directly involves labeling with SNOB reagent without using any free thiol blocker and ascorbate to reduce and acetone precipitation steps as in case of biotin switch technique.

The result in Fig. 3.22 represents that SNOB reagent reacts with both nitrosylated and non-nitrosylated GAPDH and SNOB control does not react.

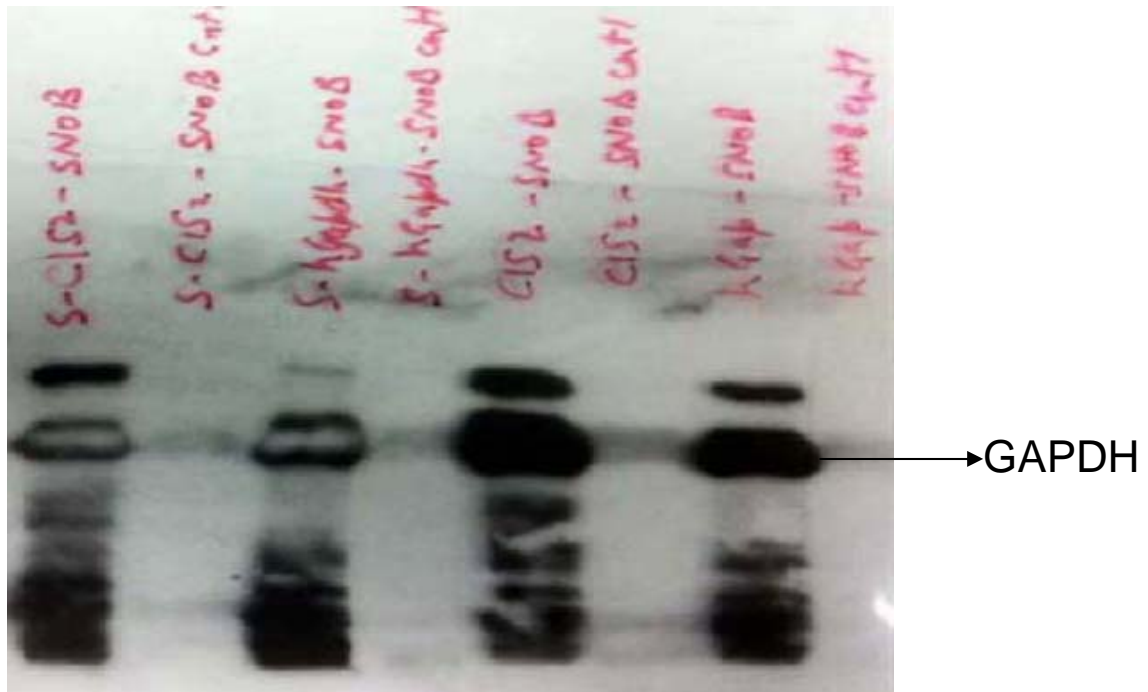


Fig.3.22. The immunoblotting representing SNOB reagent and control. Human wild type and human C152S mutant GAPDH nitrosylated and non nitrosylated were mixed with SNOB reagent and control at 60 degree centigrade and immunoblotted. The result shows that the SNOB reagent or the control has no specificity for nitrosylated and non nitrosylated GAPDH.

The result in Fig. 3.22 represents that SNOB reagent reacts with both nitrosylated and non-nitrosylated GAPDH and SNOB control does not react. Different conditions were examined, where only nitrosylated GAPDH reacts with SNOB reagent. SNOB was reacted with nitrosylated and non-nitrosylated GAPDH 60° C for 30 minutes, 40° C for 30 minutes and exposed to high intensity light for 2-3 minutes [The patent document for use of SNOB reagent suggested to test 2 temperatures]. Same concentration of protein was loaded onto each well in gel. The result below shows that SNOB technology has no specificity for nitrosylated and non-nitrosylated GAPDH.



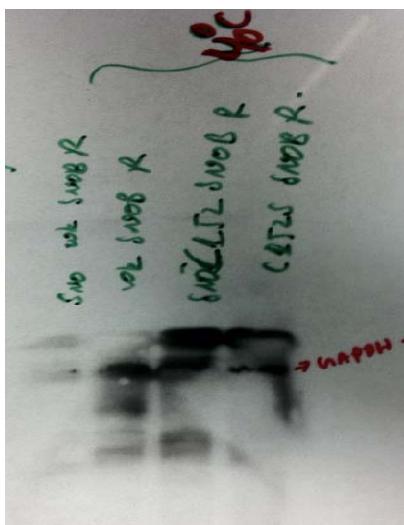


Fig.3.23. The immunoblotting representing SNOB reagent and control. Human wild type and human C152S mutant GAPDH nitrosylated and non nitrosylated were mixed with SNOB reagent and control at 3 different conditions 60 degree centigrade, 40 degree centigrade and high beam light for 2 minutes and immunoblotted. The result shows that the SNOB reagent or the control has no specificity for nitrosylated versus non nitrosylated GAPDH.

Biotin switch technique was used to identify the extent of nitrosylation on each individual cysteine with little modification; MMTS (methyl methane thiosulfonate) was replaced with NEM (N-ethylmaleimide). Biotin-HPDP was used as labeling agent; quantification of biotin-HPDP was done by mass spectrometry. Human GAPDH and rabbit GAPDH were used. The sample buffer used here is non-reducing buffer with no boiling. Same amount of sample was loaded onto each well. The controls used here was non-nitrosylated GAPDH. The gel electrophoresis was done until protein bands get separated. The gel is then subjected to mass spec analysis. Of the four cysteines in rabbit GAPDH three were identified and the cysteine at 282 was not identified. In human GAPDH 3

cysteines were identified in the Carbamidomethylated, NEM, and biotin modified form.

1.) Human GAPDH: C152, C156, and C247.

The T146-162 peptide, IISNASCTTNCLAPLAK, contains the Cys150 and Cys152 residues and was identified in several forms including the carbamidomethylated, NEM, and biotin modified form. The only biotin form of the IISNASCTTNCLAPLAK peptide identified in these experiments was the C+559 form. The C247 peptide, VPTANVSVDLTCR, was also identified in the carbamidomethylated, NEM, biotin 559, and biotin 525 forms.

-While three sites of C+Biotin were identified in these bands, ratios of the CBiotin/ (CNEM+C) may be giving a first estimation to the degree of modification at these sites. These ratios for human GAPDH Cys152:Cys156:Cys247 are 0.0021:0.0003:0.0017 indicating that the preference of NO modification is Cys152 ~ Cys247 > Cys156

2.) Rabbit GAPDH: C150, C154, and C245.

The results from the rabbit samples were very similar to the human samples with each Cys Residue identified in the C, CNEM, and CBiotin forms. While both C150 and Cys154 were identified in the biotinatelyed (559) form Cys150 appears to be modified to a greater extent than Cys154. In addition, only the Cys150 residue was identified in the CBiotin (525) form. The Cys245 residue was also identified in all four forms. Consistent with the results of human GAPDH the C+525 Da form is almost an order of magnitude lower in abundance than the C+559 Da

form. In rabbit the ratios for Cys150:Cys154:Cys245 are 0.003:0.001:0.05 indicating that modification preference is Cys247>Cys150>Cys154. The Cys282 is not identified both by trypsin and chymotrypsin digestion as the peptide is missing.

Amount of nitrosylation from mass spectrometry experiments was found to be low which when compared to extent of nitrosylation determined by Saville's method. Factors affecting amount of nitrosylation during the process were examined. The first factor which was considered was the choice of thiol blocker, MMTS and NEM. MMTS and NEM were reacted with nitrosylated GAPDH. The excess NEM or MMTS was removed by acetone precipitation. Nitrosylation was measured by saville's method before and after thiol blocker treatment. The result indicates MMTS treatment precipitates GAPDH; however NEM treatment does not precipitate protein and or loss of nitrosylation.

The next factor which was considering was biotin HPDP. Biotin-HPDP was replaced with alkylating agent iodoacetamide. The results were described below.

Human GAPDH:

The only form the Cys152, Cys156, and Cys247 peptides identified in the unmodified human GAPDH is the CNEM form, while both the C and CNEM forms were identified in the +NO samples. If we assume that all of the carbamidomethylated modified Cys residues are derived from Cys-NO and no other forms of this residue are present, than we can utilize these targeted

experiments to determine the %Cys-NO for each residue. For human GAPDH these values are 93% for Cys152, 42% for Cys156 and 14% for Cys247.

Rabbit GAPDH:

The results from the rabbit GAPDH differ from human GAPDH in that both the carbamidomethylated and CNEM forms were identified in the unmodified and +NO samples, however, the amount of the carbamidomethylated forms in the unmodified samples are small (less than 5%). At this time the source of these residues is unclear, however, these residues may represent free thiol not blocked with NEM. Despite this, the degree of modification at C150, C154, and C245 can be determined from these experiments. For rabbit GAPDH the %C-NO values are 98% for Cys150, 96% for Cys154, and 75% for Cys245. All of these values are higher than that observed for human GAPDH.



Fig. 3.24. The gel that was subjected to mass spectrometric analysis.

Distribution of C and C^{NEM} in human GAPDH^b

	Human GAPDH + NO		Human GAPDH - NO	
	%C ^a	%C ^{NEM}	%C ^a	%C ^{NEM}
C152	93%	7%	0%	100%
C156	42%	58%	0%	100%
C247	14%	86%	0%	100%

- a. C represents the NO modified cysteine, (NO → DTT → IA)
b. C^{NEM} represents unmodified C residues.

Table.3.2.The extent of nitrosylation that has occurred at 3 cysteines in human GAPDH, the result shows that c152 is the most susceptible with 93%, c156 with 42% nitrosylation and c247 with 14% nitrosylation.

Distribution of C and C^{NEM} in rabbit GAPDH

	Rabbit GAPDH + NO		Rabbit GAPDH - NO	
	%C ^a	%C ^{NEM}	%C ^a	%C ^{NEM}
C150	98%	2%	5%	95%
C154	96%	4%	4%	96%
C245	75%	25%	3%	97%

- a. C represents the NO modified cysteine, (NO → DTT → IA)
b. C^{NEM} represents unmodified C residues.

Table.3.3.The extent of nitrosylation that occurred at 3 cysteines in rabbit GAPDH as the fourth cysteine was not detectable. The amount of nitrosylation found to be same on Cys150 and Cys154 and slightly more compared to Cys245.

From the above experiment we identified that all the cysteine residues present in GAPDH underwent differential nitrosylation, in the preference order Cys152>Cys156>Cys247 in human GAPDH. In case of rabbit GAPDH S-nitrosylation is in the order of Cys150~Cys154>Cys247.

3.8 Making different GAPDH mutants and their effect on heme delivery after S-nitrosylation:

In parallel we did different point mutations, by mutating cysteine in GAPDH to further investigate whether s-nitrosylation of specific cysteine residues were involved in heme transfer to apomyoglobin. Cysteine was replaced with serine. There are 3 cysteines in human GAPDH at positions 152, 156 and 247. C152S mutant was available in the lab. We made single point mutations at cysteines 156 and 247 sites. Double mutations include 152/247, where only cysteine 156 is present, 156/247 where only cysteine 152 is present and 152/156 where only cysteine 247 is present. We made a triple mutant where all the cysteines in GAPDH were replaced by serine residues.

Once the mutations were done, yield of different transformation colonies was checked. The results were shown below. The colonies with maximum protein concentration were used for purification of different mutants of GAPDH. Purification yields vary from 5 to 15 mg per 4 litre of culture of E.coli.

Western blot of mutants: The results of experiment of the different GAPDH were given in Fig.3.25.

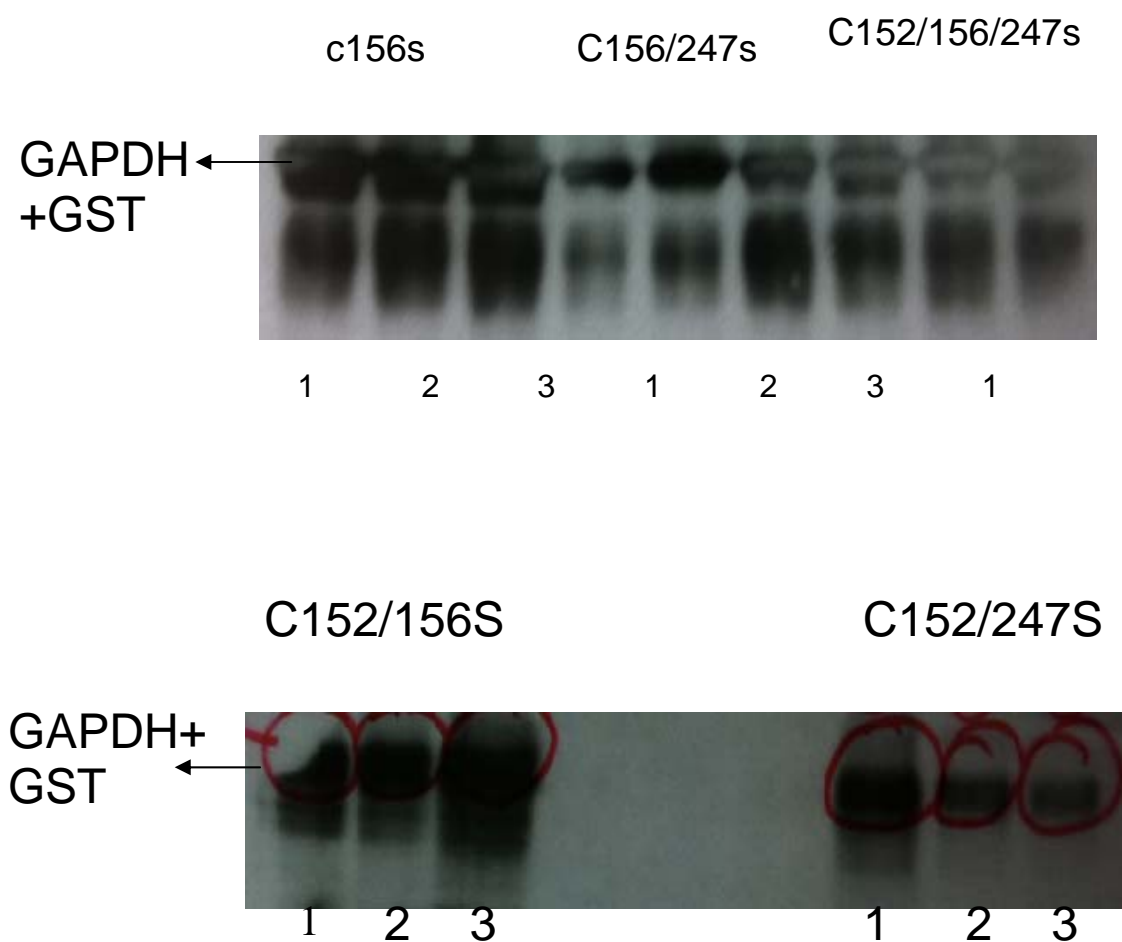


Fig.3.25. In case of cysteine 156 mutant 3 colonies expressed equally, in case of cysteine 156 247 double mutant 2nd colony has more expression, in case of triple mutant 3 colonies express equally. In case of cysteine 152/156 3rd colony has maximum expression; in case of cysteine 152/247 1st colony has maximum expression.

As described in our GAPDH purification protocol different mutants of GAPDH were purified and the purity was examined by gel electrophoresis. The result below shows that all the proteins were good in terms of purity (>85%).

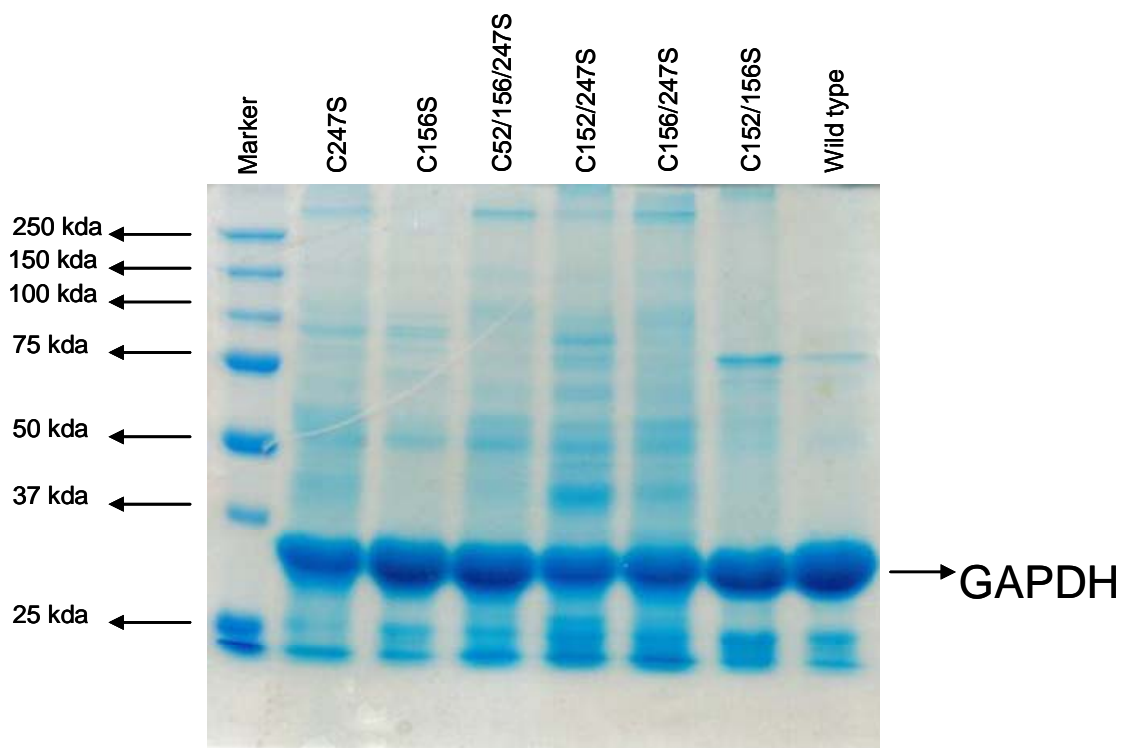


Fig.3.26. The gel represents different mutants of GAPDH, purity is ~85%.

The rate of heme transfer was examined from different mutants of GAPDH to apomyoglobin. We considered nitrosylation as a factor to check which mutant has a detectable difference on the rate of heme transfer after getting nitrosylated. By this means we can investigate which nitrosylated cysteine has an effect on the rate of heme transfer.

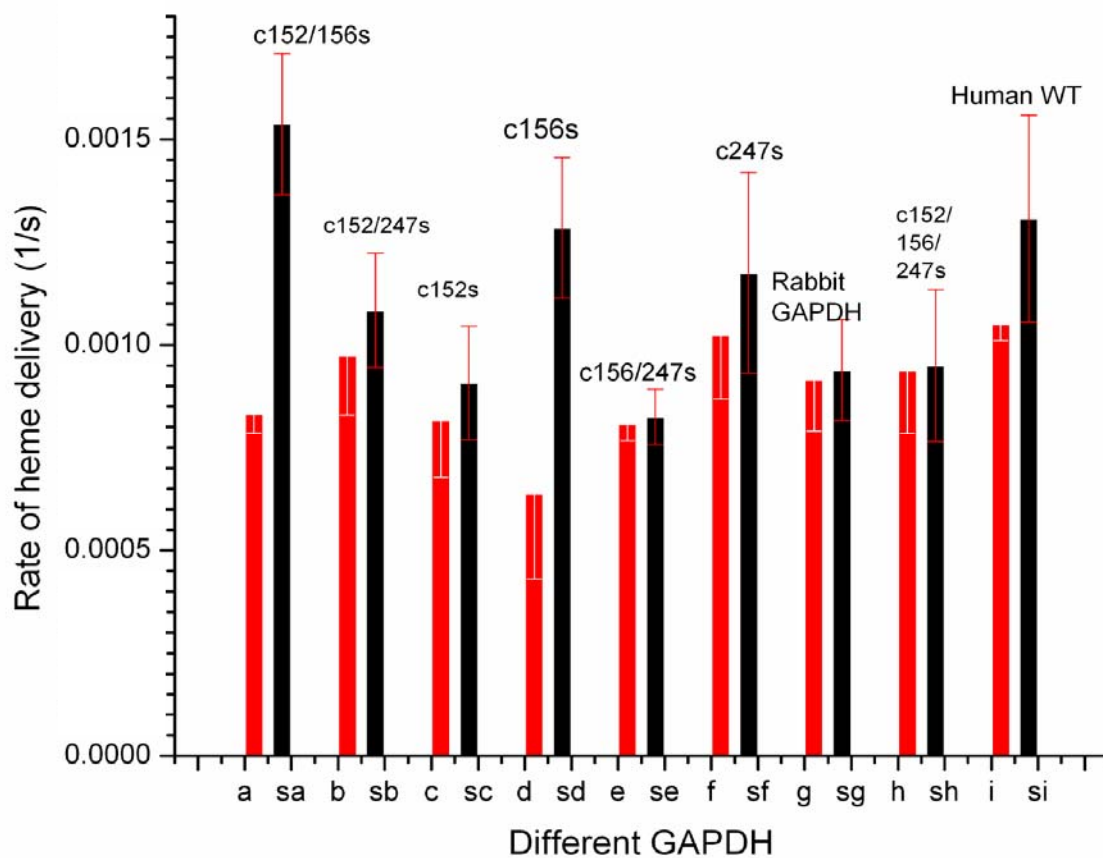


Fig.3.27. In the above graph rate of heme transfer from non-nitrosylated GAPDH was represented by red bar and black bar represents rate of heme transfer from nitrosylated GAPDH. The result suggest that there was no affect of nitrosylation on rate of heme transfer other than 2 mutants C152/156S and C156S

The above result suggests that there was no affect of S-nitrosylation on the rate of heme transfer other than 2 mutants a.) C152/156S where both cysteines 152 and 156 were replaced with serines and only cysteine 247 is present. Upon nitrosylation the rate of heme transfer doubled that of the corresponding non-nitrosylated protein but the difference is not substantial b.) C156S which has cysteines 152 and 247 also shows double the rate of heme transfer but the difference is not substantial.

CHAPTER IV

CONCLUDING REMARKS AND FUTURE INVESTIGATIONS

Based on the results presented in this thesis, we conclude that S-nitrosylation of cysteines in human GAPDH has no affect on rate of heme transfer to apomyoglobin. The factors that affect the extent of nitrosylation included dose of nitrosylation donor in case of human GAPDH and pH in the case of rabbit GAPDH (alkaline pH increased the extent of nitrosylation). Nitrosylated GAPDH was more stable at 4°C than at room temperature. The presence of reducing agents or oxygen did not affect the yield of nitrosylation. The mass spectrometry results suggest that the extent of nitrosylation on each individual cysteine is species-specific as the cysteines present in rabbit and human GAPDH's displayed different extents of nitrosylation. In case of human GAPDH the extent of nitrosylation is in order of Cys152 (93%)>Cys156 (42%)>Cys247 (14%), whereas in rabbit GAPDH the extent of nitrosylation is in order of Cys150 (98%) ~Cys156 (96%)>Cys247 (75%). From

here, there are a few topics that can be further explored in order to fully understand the mechanism of heme transfer from GAPDH.

a.) Even though we have not seen the effect of S-nitrosylation on rate of heme transfer from GAPDH, there could be other properties of GAPDH which may possibly be affected. We could explore the effect of S-nitrosylation on the binding affinity (K_{on}) of heme to GAPDH. We could explore how the protein-protein interactions of GAPDH with heme-protein differ, once the GAPDH is S-nitrosylated.

b.) We could explore how the properties of heme are affecting the rate of heme transfer after nitrosylation. This can be tested by using different heme analogues like *meso*-heme, *deutero*-heme, Zn^{+2} -heme and Co^{+2} -heme. By investigating this, we can examine whether the structural or chemical properties of heme have any effect on rate of heme transfer after nitrosylation.

c.) We could test GAPDH from other organisms to see whether the specific nitrosylation has any effect on the rate of heme transfer. This can be examined using site directed mutagenesis on cysteine residues of rabbit and bacterial GAPDH's. One could investigate whether, other organism GAPDH's behave in a similar way after nitrosylation.

d.) We have to yet to identify which aminoacids are ligating the heme. Some of our preliminary data suggest it may be histidine or cysteine. Recently, our lab has observed preliminarily heme binding is not reduced after mutating cysteines, therefore ruling out the cysteines as ligands. This can be done by mutating the

histidines and see whether these mutations has any effect on heme binding property of GAPDH. Histidine specific chemical modification can also be considered as an alternative.

The results from this work and future investigations can help better understand the mechanism of heme transfer from GAPDH to many physiologically relevant apo-protein targets such as apo-NOS, apo-cytochrome p450.

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